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Emma Tamsin

Changes in splenic microarchitecture and B cell responses in acute and chronic *Plasmodium chabaudi chabaudi* (AS) infection in mice

Submitted by

Emma Tamsin Cadman

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University College London

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I, Emma Tamsin Cadman, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

This thesis investigates the order and timing of changes in the splenic microarchitecture and the lifespan and kinetics of B cells and plasma cells during primary acute *P.chabaudi* infection, and the length of chronic *P.chabaudi* infection. Infected red blood cells can be seen in the spleen as soon as 1 hour post infection, alterations in the splenic microarchitecture begin at day 5 post-infection with the movement of dendritic cells and the merging of B and T cell zones. Macrophages and B cells of the marginal zone are lost from this location by day 10 post-infection, however there is no alteration in the location of red pulp macrophages. Large numbers of plasma cells can be seen in the spleen from day 8 post-infection, when they switch from IgM^+ to IgG^+. The adhesion molecule MAdCAM-1 is more widely expressed, although not on reticular fibroblasts or red pulp endothelial cells. The significance of these alterations, and the mechanisms responsible, are discussed. Chronic, subpatent parasitaemia in *P.chabaudi* infection is cleared by 3 months post-infection in C57BL/6 mice, and 2 months in BALB/c mice.

Most changes in B cell and plasma cell formation occur during the acute infection, and are resolved by 6 weeks post-infection. The number of B cells in the bone marrow is reduced, and plasma cell production in the spleen is high during acute infection. Plasma cell migration to the bone marrow is consistently lower in infected mice than naïve mice. The majority of B cells and plasma cells produced throughout the acute and chronic infection are short-lived, with little evidence of production of long-lived cells. B cell and plasma cell formation is still elevated at 12 weeks post-infection, indicating that B cells and plasma cells during this period may be maintained by continuous turnover rather than production of long-lived cells.

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Publications

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Achtman AH, Cadman E, Stephens R, Harrison V & Langhorne J: *B cell longevity after malaria infection in the mouse: Analysis of B cell longevity and parasite persistence after malarial infection.* (manuscript submitted)

Table of Contents

Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Summary

Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Abbreviations

ADCI	Antibody-dependent cellular inhibition
AP	Alkaline phosphatase
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor belonging to the TNF family
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BCR	B cell receptor
BrdU	5-bromo-2-deoxyuridine
B_{reg}	Regulatory B cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CGG	Chicken gamma globulin
CTL	Cytotoxic lymphocyte
DAB	Diaminobenzidine tetrachloride
DC	Dendritic cell
dH_2O	Distilled water
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
FACS	Fluorescence activated cell sorting
Fc	Crystallisable fragment
FCS	Foetal calf serum
FcR	Crystallisable fragment receptor

FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GC	Germinal centre
GDP	Gross domestic product
GPI	Glycosylphosphatidyl inositol
HBSS	Hank's buffered salt solution
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HVEM	Herpes virus entry mediator
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intra peritoneal
i.v.	Intra venous
LCMV	Lymphocytic choriomeningitis virus
LIGHT	Homologous to lymphotoxin, inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed on T lymphocytes
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTβR	Lymphotoxin β receptor
MAdCAM-1	Mucosal addressin cell adhesion molecule-1

MBP	Maltose binding protein
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein-1
MMM	Marginal metallophilic macrophages
MSP-1	Merozoite surface protein-1
MZ	Marginal zone
MZM	Marginal zone macrophages
NBT	Nitro-blue tetrazolium
NK	Natural killer
NP	(4-hydroxy-5-nitrophenyl) acetyl
NP-40	Nonylphenyl-polyethyleneglycol acetate
OVA	Ovalbumin
PALS	Periarteriolar lymphatic sheath
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PNA	Peanut agglutinin
pRBC	Parasitised red blood cell
PRR	Pattern recognition receptor
Rag	Recombinase activating gene
RBC	Red blood cell
RP	Red pulp
rRNA	Ribosomal ribonucleic acid
S_1P	Sphingosine-1-phosphate

SAv	Streptavidin
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SEMS	Single epitope multi staining
SIGNR1	Specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin related-1
SSC	Side scatter
TAP	Transporter associated with antigen processing
T1	Transitional type 1
T2	Transitional type 2
Tc	Cytotoxic T cell
TCR	T cell receptor
TD	T cell dependent
TGF	Tumour growth factor
Th	T helper cell
TI	T cell independent
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tr	T regulatory cell
T_{reg}	Regulatory T cell
WP	White pulp
YEPD	Yeast extract peptone dextrose

Chapter 1

Introduction

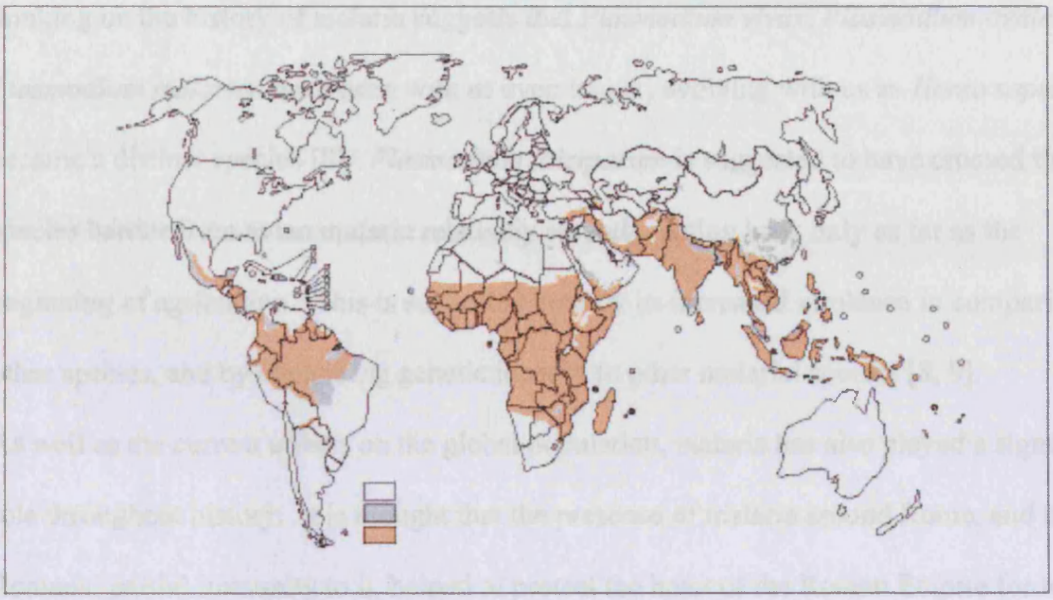
Malaria today

Malaria is one of the most prevalent transmissible human diseases, with around 300 million infections and 1 million deaths annually, with the majority of those deaths being children between the ages of 6 months and 5 years living in endemic areas [1]. Endemic areas include sub-Saharan Africa, south east Asia and the Indian subcontinent, Central America and northern parts of South America (Figure 1A). Of those areas, the highest transmission intensity is in central Africa (Figure 1B). It is no accident that the countries most affected by malaria are some of the poorest countries in the world. Malaria is currently estimated to restrict economic growth by up to 1.3% per year [2], and is estimated to cost African countries up to $12 billion per year in lost GDP [3], even though the provision of mosquito nets is a cheap and easy way to reduce the incidence of disease [4].

History of malaria

The name malaria comes from the Italian *male d'aria* or *bad air*, so called because of its association with noxious smelling swamp land that provided good breeding grounds for the mosquito vector. The disease acquired this name sometime in the 16th century, becoming *mal'aria* in the 17th century and finally dropping the apostrophe in the early 19th century to become the word we know today. Despite this relatively recent nomenclature, malaria has plagued mankind for millenia. Physical evidence of malaria infection such as enlarged spleens of Egyptian mummies and anaemia in human bones, as well as literary references

A)



Above: World malaria situation. Malaria is endemic to tropical and subtropical regions.

B)

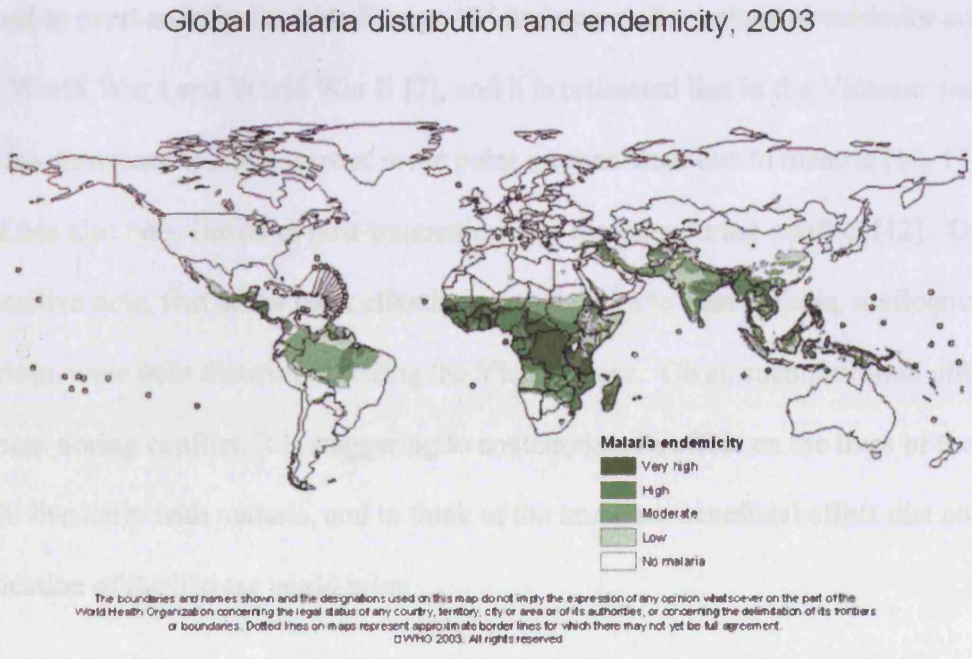


Figure 1. World incidence of malaria (A) and transmission intensity (B)

From the World Health Organisation [5, 6]

from Chinese and Mesopotamian medical writings, goes back to 4000 B.C. [7]. Modern thinking on the history of malaria suggests that *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* have been with us even longer, evolving with us as *Homo sapiens* became a distinct species [8]. *Plasmodium falciparum* is suggested to have crossed the species barrier from avian malaria relatively recently, dating back only as far as the beginning of agriculture. This is suggested both by its increased virulence in comparison to other species, and by comparing genetic identity to other malarial species [8, 9].

As well as the current effects on the global population, malaria has also played a significant role throughout history. It is thought that the presence of malaria around Rome, and the Romans' partial immunity to it, helped to protect the heart of the Roman Empire for many years [7]. The incidence of malaria decreased in Europe over time, in all likelihood due to increased use of the land leading to loss of mosquito breeding grounds, however it continued to exert an effect outside Europe. Malaria created a serious obstacle for armies in both World War I and World War II [7], and it is estimated that in the Vietnam war up to half of the American soldiers were at some point incapacitated due to malaria [10, 11]. Malaria has also been linked to post-traumatic stress disorder during conflict [12]. On a more positive note, two of the most effective current drugs to treat malaria, mefloquine and artemisinin, were both discovered during the Vietnam war. Given such profound effects of the disease during conflict, it is staggering to contemplate its effect on the lives of those who still live daily with malaria, and to think of the immense beneficial effect that control or eradication of the disease could bring.

Biology of malaria

The malarial disease is caused by a Protozoan parasite of the genus Plasmodium. The four species that infect humans are *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum*, with *P. falciparum* being responsible for the most severe forms of the disease. The Plasmodium parasite was first discovered in 1880 by Alphonse Laveran, with its cyclical development and rupture of red blood cells associating with fever being described by Camillo Golgi in 1885. Although non-erythrocytic forms were described in 1893, it was not until 1948 that they were shown to develop in liver cells. Transmission of the parasite by mosquitoes was demonstrated by Ronald Ross in 1897, and the fourth species of human parasites, *Plasmodium ovale*, was identified in 1922 with its life cycle completed in 1955 [7] (Figure 2).

Human malaria is only transmitted by female mosquitoes of the *Anopheles* genus, with *Anopheles gambiae* being considered the most efficient vector [13]. Other species of malaria can be transmitted by mosquitoes of a different genus, including *Mansonia* and *Aedes*. *P. falciparum* is capable of developing in *Mansonia* mosquitoes, however natural transmission with this vector has never been demonstrated (review [14]). On feeding, the mosquito injects an estimated 25-50 sporozoites per infective bite [15], however some of these remain in the dermis and some migrate through the lymphatic system to the lymph node, where they are degraded [16]. Sporozoites that reach the blood stream migrate to the liver where they undergo a 5-15 day replication phase in human malarias [15], 42-72 hours in rodent malarias [17], before releasing merozoites back into the blood. *P. vivax* and *P. ovale* can both persist as hypnozoites, a dormant phase in the liver, re-emerging months or years after clearance of blood-stage parasites as can the primate malaria *P. cynomolgi*

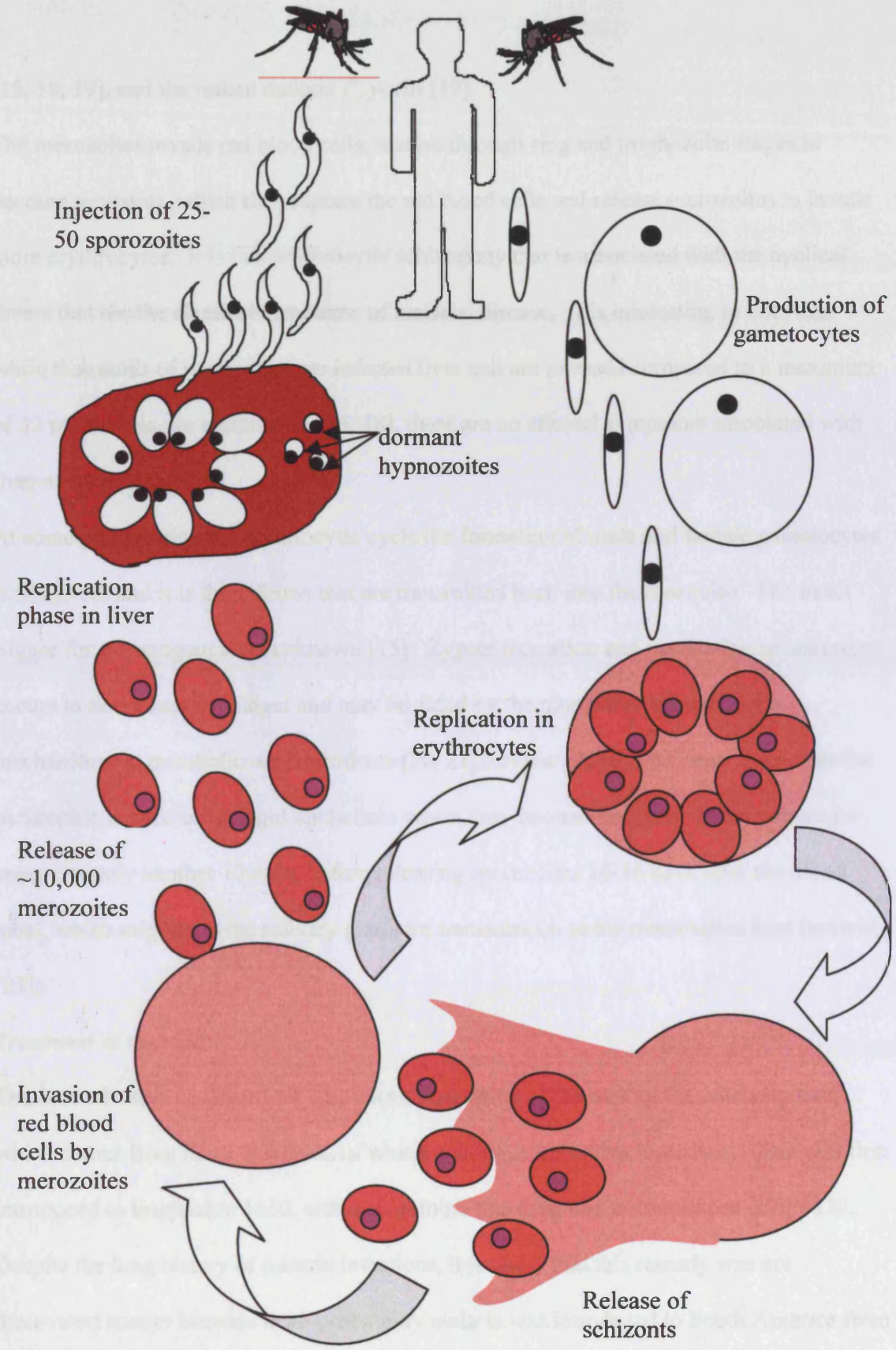


Figure 2. Generalised Plasmodium life cycle in mammals

[15, 18, 19], and the rodent malaria *P. yoelii* [17].

The merozoites invade red blood cells, mature through ring and trophozoite stages to become schizonts, which then rupture the red blood cells and release merozoites to invade more erythrocytes. It is this erythrocytic schizogony that is associated with the cyclical fevers that are the classical symptoms of malarial disease. It is interesting to note that while thousands of merozoites per infected liver cell are released compared to a maximum of 32 merozoites per erythrocyte [15, 18], there are no clinical symptoms associated with liver-stage schizogony.

At some point during the erythrocytic cycle the formation of male and female gametocytes is triggered, and it is these forms that are transmitted back into the mosquito. The exact trigger for gametogenesis is unknown [15]. Zygote formation and maturation to ookinetes occurs in the mosquito midgut and may be aided by the mosquitos' own defence mechanisms or metabolic waste products [20, 21] (review [22]). Ookinetes then cross the peritrophic matrix and midgut epithelium where they become oocysts. These mature for approximately another 10 days, before releasing sporozoites 14-16 days after the blood meal, which migrate to the salivary gland for transmission to the mammalian host (review [23]).

Treatment of malaria

The first effective treatment for malaria was ingestion of the bark of the cinchona tree, which comes from South America, of which quinine is the active ingredient. This was first introduced to England in 1650, although quinine as a drug was not produced until 1820. Despite the long history of malaria infections, it is likely that this remedy was not discovered sooner because in all probability malaria was introduced to South America from

Europe. The introduction of malaria to South America by the slave trade is suggested by the lack of genetic mutations protecting against malaria in the native South American genome, as well as by the limited number of parasites and vectors in comparison to Asia, the purported origin of malaria [7, 24]. The first synthetic anti-malarial drug was chloroquine, produced in Germany in 1937 [7]. It is still in use today, although growing parasite resistance has limited its use. Many other anti-malarial drugs have since been produced, including mefloquine (Larium™), atovaquone/proguanil (Malarone™), doxycyline, primaquine and artemisinin [25], however resistance to these is also continually developing.

Models of malaria

The main barriers to the study of malaria in the human population are the availability of appropriate tissue samples, most studies having access only to peripheral blood, and the lack of control over timing of infection and re-infection. Although it is possible to plan careful studies around transmission seasons, there will always be certain questions that cannot be answered by a study of human infections.

The closest model system to humans is primates. The natural non-human hosts of malaria in Asia are largely primates of the genus *Macaca*, which are infected by *P. inui inui*, *P. cynomolgi cynomolgi*, *P. c. bastianelli*, *P. coatneyi*, *P. i. shortii*, *P. knowlesi*, *P. k. edesoni*, *P. fragile*, *P. semiovale*, *P. fieldi* (review [26, 27]). Addition species in Asia, *P. hylobati* and *P. eylesi* are parasite of gibbons (*Hylobates*) [14]. In Africa, *P. gonderi* infects the *Cercocebus* (mangabeys) and *Mandrillus* (drill) primates, *P. reichenowi* and *P. rodhaini* infect the chimpanzees *Pan satyrus* and *Pan troglodytes*, whilst in South America *P.*

brasilianum infects *Cebus, Ateles, Saimiri, Lagothrix, Alouatta* and *Cacajao* genii and *P. simium* infects the howler monkeys (*Alouatta fusca*) [9, 24].

P. knowlesi and *P. cynomolgi* in rhesus monkeys (*Macaca mulatta*) are the best studied primate malarias. The rhesus monkeys are not a natural host for these *Plasmodia* and therefore the infection is much more severe than in the natural host, *P. knowlesi* being a lethal infection in this rhesus monkeys [26]. *P. knowlesi* has been used as a model for cerebral malaria, whilst *P. cynomolgi* has been used as a model for *P. vivax*. These models do not exactly replicate the human infection however, rhesus monkeys show little sign of illness during infection even with high parasitaemias, despite the ultimately fatal outcome of infection [26]. Due to difficulties obtaining primate hosts and the ethical considerations involving the use of primates for experimentation, other models have been more widely utilised although some work on malaria in non-human primates continues [28].

Prior to the discovery of rodent malarias, the alternative models to primate malarias were avian malarias. The mostly widely used avian malaria model is *P. gallinaceum* in chicks, which was discovered in 1935 [29], others include *P. relictum* and *P. cathemerium* in canaries, *P. lophurae* in ducks *P. circumflexum* and *P. elongatum* in sparrows [17, 29].

Rodent malarias were first discovered as sporozoites during dissection of *Anopheles dureni millecampsi* by Vincke and Comte d'Ursel in 1943. Entomological studies in the following year revealed that this mosquito fed on animals, and in 1948 malaria parasites were isolated from one of their their natural hosts, the African thicket rat *Grammomys surdaster* by Vincke and Lips [17]. Although *Plasmodium* spp. are thought to have been introduced to the Americas, the fact that they are found only in African, and not in Asian, rodents is intriguing. The first described was *P. berghei*, followed by *P. vinckei* in 1952, *P. chabaudi*

in 1965 and *P. yoelii* was described as a polytypic species encompassing three subspecies of *P. berghei*-like parasites in 1974. *P. yoelii* was for a long time considered a subspecies of *P. berghei*, but is now classified as a separate species [17]. Work on these species was limited however, until the successful establishment of the first *A. d. millecampsi* and *G. surdaster* colonies in 1963 [17]. Laboratory mice are permissive hosts for rodent malarias, however mice are not their natural hosts and therefore the course of infection can be expected to be somewhat different. For example, the number of schizonts produced per merozoite is reduced in mice compared to the natural host. Likewise, other vectors such as *A.stephensi* may transmit malaria in a laboratory environment although invasion of sporozoites into the salivary glands is reduced [17].

In immunologically intact mice malaria can be either a lethal or a self-limiting infection. *P.yoelii* 17XNL produces sterile immunity after a single infection, and a secondary infection of *P.c.chabaudi* (AS) produces a much reduced peak parasitaemia of only 1% or less infected erythrocytes [30] (Figure 3B). This is in contrast to human malaria, where repeated infections are required to produce immunity [18], and to the natural host, which has recurring recrudescences of parasitaemia throughout life [17]. In addition to the differing memory responses, higher parasitaemia is required to produce clinical symptoms of disease in mice than in humans [13]. Many aspects of malaria infection can however be studied perfectly adequately in laboratory mice.

Infections in the natural host would be more similar to human infection, however such work has been limited due to the difficulty of maintaining thicket rat colonies and the lack of reagents available. Even in the natural host, in an experimental environment infections are limited to less than 6 months, possibly due either to the lack of re-infection, or to this

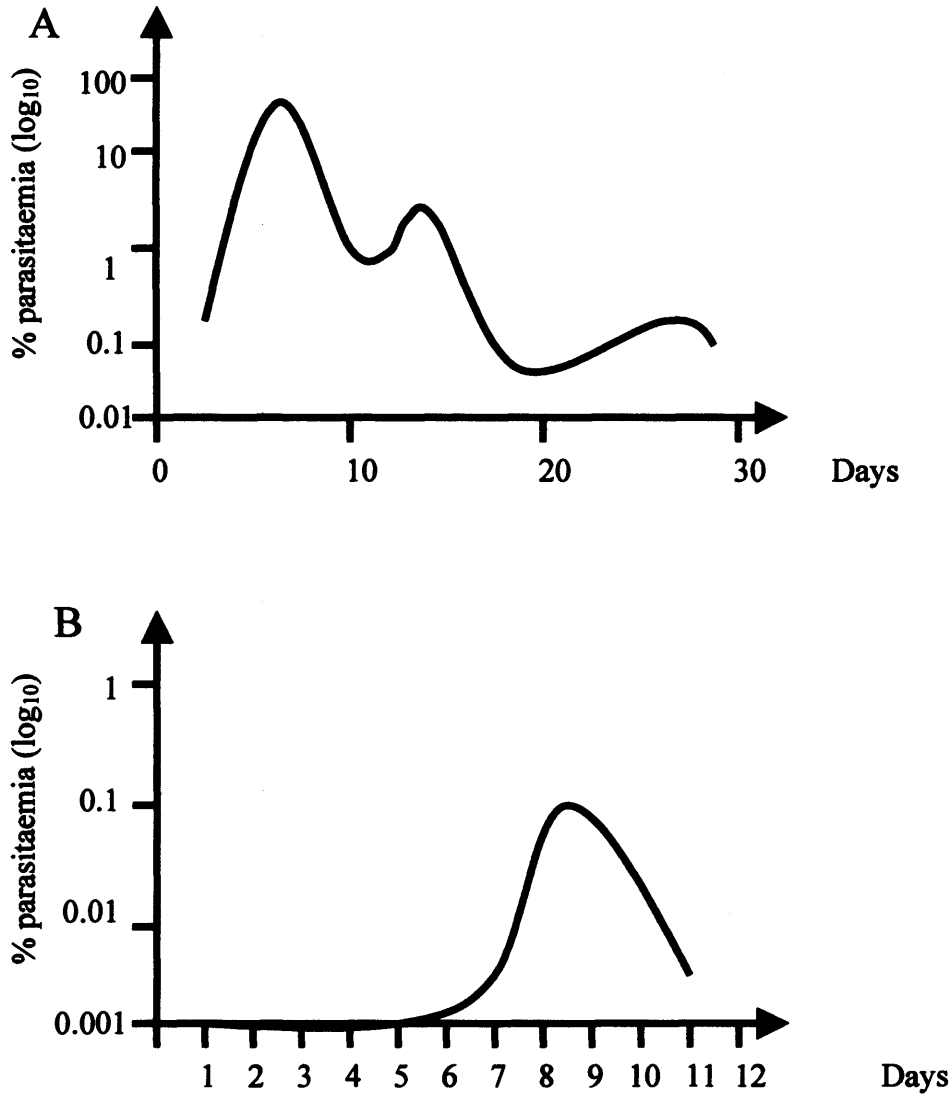


Figure 3. Diagrammatic illustration of a typical course of infection with *Plasmodium chabaudi chabaudi* (AS)

A) Primary infection with 10^5 pRBC in C57BL/6 mice

B) Secondary infection with 10^5 pRBC in C57BL/6 mice

environment discouraging the development of liver hypnozoites [17]. For a thorough investigation of chronicity of infection, the natural host would still be the best. For example, temperature has been shown to affect many aspects of the malaria life cycle. The development of oocysts and sporozoite infectivity may be reduced if the temperature the mosquitoes are kept at is either too low or too high. Likewise, cold temperatures have been used to induce the development of exoerythrocytic schizonts, which may be hypnozoites [17].

The three main rodent malaria species used in laboratory studies today are *Plasmodium chabaudi*, *Plasmodium yoelii*, and *Plasmodium berghei*. These can be further divided into subspecies, for example *Plasmodium chabaudi chabaudi* and *Plasmodium chabaudi adami*. Of these species there are different strains such as *P.c.chabaudi* (AS), (AJ), (CB), (ER) and others [31]. Classification of malaria parasites has been difficult as species that we now recognise as distinct are difficult to distinguish under the microscope. The classical definition of a species is a group that is reproductively isolated yet, as *Plasmodium* sexual reproduction occurs in the mosquito vector, this is difficult to test. Early classification was further hindered by the fact that many isolates were in fact mixed infections of different species, as are most natural infections [17]. Much of the current classification comes from examination of isoenzymes, and it has been suggested that rodent malaria species are still in active evolution [17]. As the African landscape continues to change, geographical barriers may occur to hasten speciation.

Strains differ in virulence and in lethality, for example *P.c.chabaudi* (ER) being more virulent than *P.c.chabaudi* (AJ), which is in turn more virulent than *P.c.chabaudi* (AS) [31]. *Plasmodium berghei* (ANKA) is used as a model for cerebral malaria [15]. Virulence

also depends on the host, with *P.c.chabaudi* being a self-limiting infection in C57BL/6 mice, but lethal in A/J mice, with resistance to death being associated with 4 major susceptibility loci (review [32]) [33-36]. Virulence is also affected by gender, specifically testosterone levels, with female mice being more resistant than male mice [37, 38].

The model system used in this study is *P.c.chabaudi* (AS) in female C57BL/6 and BALB/c mice, the natural host being *Thamnomys rutilans* [17]. The course of infection in both mouse strains is almost identical, although BALB/c mice have an increased tendency to die at higher doses of infection (10^7 parasitised red blood cells, unpublished observations). The common infective dose used in our laboratory is 10^5 parasitised red blood cells (pRBC) and a typical course of parasitaemia in C57BL/6 mice is shown in Figure 3A. Peak parasitaemia of 20-30% occurs around day 8 of primary infection with an infective dose of 10^5 pRBC, around day 7 with an infective dose of 10^6 pRBC, day 9 for infection with 10^4 pRBC, etc. [39]. Parasitaemia falls below detection limits around day 30 in *P.c.chabaudi* infection, with *P.yoelii* infections being eliminated earlier [40]. Chronic, low-level parasitaemia persists for some weeks before final clearance. In a secondary infection, parasitaemia becomes visible around day 5, with peak parasitaemia of less than 1% occuring around day 8, falling below detection limits around day 12 (Figure 3B) [41].

The immune system

The immune system can be divided into two parts, the innate immune system and the adaptive immune system. The innate immune system is considered to be the ancestral immune system, from which the adaptive immune system evolved. The main difference between them is that the responses of the innate immune system remain the same on

subsequent encounters with the same pathogen. By contrast, the adaptive immune system is capable of memory responses [42].

The innate immune system is the first line of defence against foreign bodies. It consists of anatomical barriers (e.g. skin), physiological barriers (e.g. non-permissive temperature, gastric acid), phagocytes (e.g. macrophages), leukocytes (e.g. granulocytes and NK cells) and serum factors (e.g. complement, acute phase proteins, histamine and antibacterial proteins) [42, 43]. The innate immune system works in concert with adaptive immunity, providing antigen presentation and cytokine signals. Conversely, the adaptive immune system helps the innate immune system, for example by antibody opsonisation of antigens for phagocytosis. The adaptive immune system can be subdivided into humoral (antibody-mediated) and cellular immunity. The components of the adaptive immune system are lymphocytes – B cells and T cells.

There are two methods of distinguishing pathogens. The first is recognition of specific pathogen ligands or pathogen-related molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as protein kinase R, C-type lectins (review [44]) and Toll-like receptors (TLRs) [45]. The Toll receptor was first discovered in Drosophila by Hashimoto, Hudson & Anderson in 1988 [46]. TLRs were subsequently discovered in humans in 1997 by Medzhitov, Preston-Hurlburt & Janeway [47], and recognise molecules such as bacterial endotoxin (LPS) [48], bacterial and viral DNA and RNA [45, 49] (review [50]). Such receptors are considered to be the bridge betweeen the innate immune system and the adaptive immune system [51]. The expression of multiple TLRs and convergent signalling pathways allows for increased flexibility in the cell's response, and cytokines produced by other cells may also modulate the expression of TLRs [50].

TLRs recognise distinct molecular signatures from pathogens and form a basis for targeting specific immune responses to specific infections [45]. TLR ligation can induce dendritic cell maturation and up-regulation of the co-stimulatory molecules necessary to induce T cell activation [45]. Dendritic cell maturation can also be triggered by cytokines, however this is not full activation, and does not lead to full activation of T cells. PRR signalling is still required for priming of a T cell response [52]. Although TLRs are expressed on many different cell types, their function has been best studied in dendritic cells. Such studies are complicated however, because TLR function is not always conserved between human and mouse, expression patterns differ and not all TLRs are currently known to have functional homologues in the other species (Table 1) [45, 53].

The other method of antigen recognition is recognition by cells of the adaptive immune system. Foreign antigenic peptides are recognised by T cells when the peptides are associated with self major histocompatibility complex (MHC) molecules, of which there are three classes. MHC Class I molecules are expressed on nearly every nucleated cell type and present antigens from inside the cell, while MHC Class II antigens are expressed only on antigen-presenting cells and present exogenous antigens that have been endocytosed [43]. The MHC is also referred to as the human leukocyte antigen (HLA) complex in humans, and the histocompatibility (H-2) complex in mice. MHC genes are present on chromosome 6 in humans and chromosome 17 in mice. They code for MHC Class I molecules of three major subgroups (HLA A, B, C in humans, H-2 K, D & L in mice) and MHC Class II molecules of 2-3 subgroups (HLA DR, DP & DQ in humans, H-2 A & E in mice) [43]. Ancestors of MHC molecules can be found as far back in evolution as worms, crustaceans and insects [42].

Table 1. Expression of Toll-like receptors on dendritic cell subsets in human and mouse

	Human blood mononuclear cells		Mouse spleen dendritic cells			
	$CD11c^+$	pDC	$CD4^+CD8^+CD11c^+$	$CD8^+CD4^-CD11c^+$	$CD4^-CD8^-CD11c^+$	pDC
TLR1	+++	++	++	++	++	++
TLR2	++	+/-	++	++	++	++
TLR3	+++	-	+	+++	++	+/-
TLR4	++	-	+	+	+	+
TLR5	++	+/-	+++	+	++	++
TLR6	++	+	+++	++	++	++
TLR7	+	++	++	-	+	+++
TLR8	+	-	++	++	++	++
TLR9	-	+++	++	++	++	+++
TLR10	++	+/-	ND	ND	ND	ND
TLR11*	-	-	+/?	+/?	+/?	ND

Adapted from [45]
ND = not determined
*Human TLR11 contains stop codons that are thought to produce a truncated, non-functional protein. TLR11 is expressed on mouse dendritic cells, however its expression on specific subsets has not been defined.

Proteins from within the cell are degraded in the proteasome into small peptides (8-13 amino acids), transported into the endoplasmic reticulum by the TAP transporter protein, where they are loaded into the antigenic groove of MHC Class I molecules that are then transported to the cell surface for antigen presentation [43]. These MHC-peptide complexes are recognised by $CD8^+$ T cells, which form a surveillance system throughout the body. Presentation of foreign (e.g. viral or tumour) antigens in the context of MHC Class I leads to the activation of $CD8^+$ T cells and their differentiation into effector cytotoxic lymphocytes (CTLs). These then proceed to kill the infected cell by activation of death receptors or release of cytotoxic mediators such as perforin [43].

Specialised antigen-presenting cells (APCs) also express MHC Class II molecules. APCs take up foreign antigens and break them down to peptides of 13-18 amino acids in endosomes and lysosomes [43]. These endosomes then fuse with vesicles containing MHC Class II molecules, which are transported to the cell surface for presentation to $CD4^+$ T cells. Recognition of a wide variety of antigens is achieved by a large number of alleles of MHC genes, with polymorphism mainly within the peptide binding site, which interact with T cell receptors that undergo gene rearrangment to generate diversity during T cell development [43]. Activation of naïve T cells, however, requires engagement of co-stimulatory receptors by molecules on the surface of antigen presenting cells, as well as MHC-peptide complex recognition. Without appropriate stimulation by both of these signals, T cell anergy or apoptosis can be induced instead of activation (review [54]). Unfortunately for the immune response to malaria, mature red blood cells do not express any MHC molecules, therefore recognition of malarial antigens requires the phagocytosis of pRBCs, and the presentation of malaria antigens on MHC Class II molecules.

MHC Class III molecules, while being encoded within the third region of the MHC, are secreted molecules that have no role in antigen presentation. They do however have roles in the immune response, as they include components of the complement pathway, tumour necrosis factor α (TNFα) and lymphotoxin α (LTα) [43].

Dendritic cells

Dendritic cells (DCs) are the only antigen-presenting cells responsible for presenting antigens to naïve T cells. B cells and macrophages are also capable of antigen presentation, however they present more efficiently to antigen experienced rather than naïve T cells. DCs are required to initiate an acquired immune response, and are also involved in the early innate immune response (review [55]) [56]. They are highly phagocytic cells that constitutively take up antigens, process them into peptides in the endosome and then present those peptides to T cells in the context of MHC Class II (review [54]). Different types of DCs are found throughout the body, some residing in tissues and others recirculating in the blood and lymphatic system [55]. When immature DCs in peripheral tissues encounter antigen they migrate through the blood and lymphatic system to secondary lymphoid organs, upregulating co-stimulation molecules and MHC molecules [55, 57]. Maturing DCs also undergo conformational changes, acquiring characteristic long, branching processes known as dendrites [55, 58]. Mature DCs present antigen to T cells, thereby initiating the adaptive immune response [58, 59].

Some DCs are resident within secondary lymphoid organs. In the mouse spleen, immature or non-activated DCs are found mostly in the MZ and red pulp, with a few mature DCs in the T cell zone [60, 61] (Chapter 4, Figure 23). Upon encounter with antigen, DCs migrate into the T cell zone for antigen presentation to T cells [45, 62]. DCs are defined by their

expression of a panel of cell surface molecules, many of which are not exclusive to DCs and their expression levels may also alter depending on the activation state of the cell. There are three main types of dendritic cells in mice, which are derived from haematopoietic precursors; the Langerhans cells in the skin, the conventional DCs and plasmacytoid DCs in the blood and lymphoid organs. Most types of DCs express the cell surface molecule CD11c (α integrin associated with β_2 integrin CD18), although levels of expression vary. The main DC types can be further divided into subsets based on expression of other cell surface molecules, although it is not yet clear which of these subdivisions have functional significance [63].

Langerhans cells are present in the dermis, where they phagocytose antigens. Upon antigen encounter, they migrate to the draining lymph node where they present antigens to T cells [55]. Langerhans cells may have a role in immunity to malaria sporozoites which traffic through the skin. Whilst immunity to pre-erythrocytic stages may have a substantial effect on the development of blood-stage malaria, Langerhans cells have not been investigated in this thesis and will therefore not be discussed further.

There are two main subsets of conventional DC in the mouse spleen, distinguished by expression of CD8. These can be further subdivided by expression of CD4, CD205 and CD11b [64] (Table 2). Human DCs express CD4 but not CD8, and although they express varying levels of other surface markers, distinct populations comparable to mouse $CD8^+$ and $CD8^-$ subsets have not yet been shown [64].

Some researchers have proposed that $CD8^+$ and $CD8^-$ subsets are part of a single population at different stages of development [65], whilst others maintain they are discrete populations [66]. Both $CD8^+$ and $CD8^-$ DCs can be derived from either common lymphoid or myeloid

Table 2. Cell surface molecules used for identification of dendritic cell subsets

Cell surface molecules expressed	Dendritic cell populations											
	Langerhans cells		Plasmacytoid dendritic cells			Conventional dendritic cells						
	Human	Mouse	Human	Mouse		Human			Mouse			
CD11c	+/-	int	+/low	low	low	+	-	+	+	+	+	
CD8	-	-	-	+/-					+	-	-	
CD4	+	+	+/-	+/-					-	-	+	
CD11b	-	-	-	-		+		-	-	+	+	
CD14	-	-	-			+	-	-				
CD13			-			+						
CD33			-			+						
CD34	+			-		+	+					
Lin			-				-					
IL-3Rα	+	-	+	-		-	+					
CD1	+		-									
120G8				+								
BDCA-2			+									
BDCA-4			+									
PDCA-1				+								
CD45 RA	+	+	+/-	+								
CD45 RB		+							-	-	-	
CD45 RO						+		low				
Ly6C				+	-							
DEC-205		+/-				+			+			
Langerin	+	+										

Compiled from [49, 55, 64, 67, 68]
Blank spaces indicate not determined

progenitors (review [64]) [69, 70], although a larger number of myeloid progenitors mean that most DCs are derived from this line [69]. The $CD8^+$ and $CD8^-$ DC subsets are, however, still considered to be separate developmental lineages (review [71]). The existence of regulatory DCs ($CD11c^{low}$) has recently been described, although these are not yet well characterised and may be further subdivided into functionally distinct groups [53, 72, 73].

There has been much debate as to whether subtypes of DCs are pre-programmed to induce a particular type of immune response [74, 75] (review [76, 77]). Recent work, however, has shown that DCs exposed to two different antigens concurrently can induce separate immune responses to each antigen [78]. Current thinking suggests that DCs are a very plastic population and that each subtype may induce any type of immune response, although certain DC subtypes may have a predisposition towards inducing a particular type of immune response. The immune response they induce is highly dependent upon the type and strength of signal they receive and the local cytokine environment where T cell activation takes place [79, 80] (review [64]). This does not, however, preclude DC subsets from having differing roles in the induction of immune responses. For example, $CD8^+$ DCs are thought to specialise in the phagocytosis of apoptotic cells and the cross-presentation of antigens from these cells [63].

In addition to antigen-presentation on MHC Class II molecules, APCs are also capable of presenting exogenous antigens on MHC Class I molecules, a phenomenon termed cross-presentation. There are thought to be two mechanistic pathways for cross-presentation, one involving TAP-dependent transport of exogenous antigens out of endosomes and into the cytosol, the other involving fusion of phagocytic vesicles with vesicles containing MHC

Class I molecules (review [81]). This process would enable the activation of CTLs specific for foreign antigens. Both the $CD8^+$ and $CD8^-$ dendritic cell subsets of APCs are believed to be capable of cross-presentation, although only one subset ($CD8^+$) is suggested to do so constitutively [82]. Macrophages can also cross present antigens, however they do so with much lower efficiency than dendritic cells (review [54]).

Plasmacytoid DCs (pDCs) express low levels of CD11c and do not express CD11b (Mac-1, α integrin partner of β_2 integrin). Human pDCs also express CD4, the CD45 antigen CD45RA and the IL-3 receptor CD123 ($CDllc^{low}CD11b^-CD4^+CD45RA^+CD123^+$). Mouse pDCs do not express those molecules, but instead express the CD45 antigen B220 and the Ly6C molecule ($CD11c^{low}CD11b^-B220^+Ly6C^+$). Human pDCs specifically express the cell surface molecules BDCA-2 (a C-type lectin) and BDCA-4 (neuropilin-1) (review [83]). Mouse pDCs can also be specifically identified by the monoclonal antibodies 120G8 or PDCA-1, the ligands for which are currently unknown [80].

pDCs can be derived from either common lymphoid or myeloid progenitors under the influence of the cytokine Flt-3 ligand (review [83]) [84]. pDCs are immature cells that become dendritic cells upon antigen encounter. They are less phagocytic and less efficient at antigen presentation to naïve T cells than conventional DCs, but can promote efficient proliferation of antigen experienced T cells [67] and induce a Th1 like response characterised by IFNγ and IL-10 production [85]. The average lifespan of pDCs outside the bone marrow is 2 weeks. pDCs are thought to be important in viral infections as they are the only cell type to produce high levels of interferon α (IFNα, 200-1,000 times more than any other cell type) in response to viruses (review [67]) [80, 86]. In keeping with this function, they express only those TLRs which are required for viral DNA and RNA

recognition – TLR7 and TLR9 [67], and are progressively lost during HIV infection [86]. Viral recognition, however, triggers differentiation as well as IFNα production [85], causing pDCs to become more efficient antigen-presenting cells and reduce production of IFNα [86]. Importantly the by-product of Plasmodium breakdown of haem, haemozoin, has been shown to activate DCs via TLR9 [87].

In addition to their production of type I IFN, pDCs have also been shown to require IFNα for full maturation and migration to the T cell zone of the spleen [80]. IFNα and β, the type I IFNs, mediate a range of anti-viral effects. IFNα can enhance cytotoxicity of natural killer (NK) cells and $CD8^+$ T cells, induce IFNγ secretion and promote plasma cell differentiation [67]. Type I IFNs have also been proposed to act as immune regulators by modulating DC function, IFNγ and IL-12 expression (review [80]). pDCs act synergistically with conventional DCs for the activation of NKT cells [88], but have also been suggested to induce the formation of $CD8^+$ IL-10 producing regulatory T cells (review [89]). Pelayo and colleagues [68] recently described two distinct subsets of pDCs in mice, distinguished by expression (or lack of expression) of recombinase-activating gene-1 (Rag-1). Only Rag-1^- pDCs produced IFNγ and IL-12, whilst only T cells stimulated by Rag-1^+ cells, however expressed IL-4. Rag-1^- cells also produced higher levels of IFNα than Rag-1^+ cells.

Dendritic cells have been shown to be important for immunity to liver stages of malaria [90], but their role in blood stage infection is still being elucidated [91]. The role of plasmacytoid DCs in malaria infection is currently being investigated.

T cells

There are three different types of T cells, αβ T cells, γδ T cells and NK T cells. The most numerous of these are the αβ T cells. αβ and γδ T cells are named for the type of T-cell receptor (TCR) chains they express, either the TCR α and β chains or the γ and δ chains, whilst NK T cells express both the TCR and NK cell-specific molecules NK1.1 and DX5. All types of T cell develop in the thymus gland.

αβ T cells are divided into two subsets based on expression of the surface markers CD4 and CD8 that, along with the associated signalling complex, are co-receptors for the T cell receptor. The T cell receptor binds to MHC-peptide complexes presented by APCs. TCRs are highly diverse and this diversity is one of the mechanisms the adaptive immune system uses in order to recognise a wide variety of antigens.

$CD8^+$ T cells are also known as cytotoxic T cells (CTLs) in their effector phase. They circulate throughout the body looking for foreign antigens presented on MHC Class I molecules. They are important for immune responses to intracellular pathogens, viruses and tumours. Upon activation they may develop into CTLs, release mediators such as perforin and granzymes, which kill target cells. They can also bind receptors on target cells (e.g. Fas ligand) to initiate apoptosis [42]. $CD8^+$ T cells can be divided into two subsets (Tc1 and Tc2) based on the expression of different cytokines, however the functional significance of these subsets is unclear [92]. $CD8^+$ T cells are crucial for immune responses against intracellular pathogens such as liver-stage malaria, Leishmania, and viruses [93, 94] (review [95-98]). Mature mouse and human RBC express only low levels of MHC Class I molecules, although immature reticulocytes express higher levels. Expression of MHC Class I molecules on reticulocytes is thought to cause reduced

virulence of reticulocyte preferring parasites (e.g. *P. vivax*, non-lethal *P. yoelii*) compared to erythrocyte preferring parasites (e.g. *P. falciparum*, lethal *P. yoelii*) [99]. In *P. chabaudi* infection, $CD8^+$ T cells appear to have little role in controlling the acute peak of parasitaemia, although they do assist in the clearance of parasites later in the infection [100].

$CD4^+$ T cells are known as helper T cells because they help B cells to produce antibodies, through contact dependent receptor-ligand interactions (e.g. CD40 on B cells with CD40 ligand on T cells) and production of cytokines. $CD4^+$ T cells also stimulate proliferation and differentiation of cytotoxic T cells, and activate macrophages to produce reactive oxygen species (ROS). T helper (Th) cells can be broadly divided into two main subsets depending on the cytokines they produce (Figure 4). Whilst both subsets produce certain cytokines (e.g. GM-CSF, IL-2, IL-3), only Th1 cells produce the inflammatory cytokine interferon γ (IFNγ) that is important for cell-mediated immune responses against intracellular pathogens (e.g. viruses, Leishmania) involving cytotoxic T cells and macrophages. Th2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-13, which are important for B cell and antibody mediated responses, immune responses to extracellular pathogens (e.g. helminths) and allergic reactions. There is some overlap in function, as production of certain antibody isotypes are associated with Th1 responses and Th2 cells can also activate macrophages [101, 102]. Whilst immune responses tend to be dominated by one or the other type of response, they are not mutually exclusive. The malaria infection is characterised by an early Th1-type response followed by a switch to a Th2-type response [28, 103, 104].

Recently a third set of $CD4^+$ T cells have been described, IL-17 producing T cells, that are

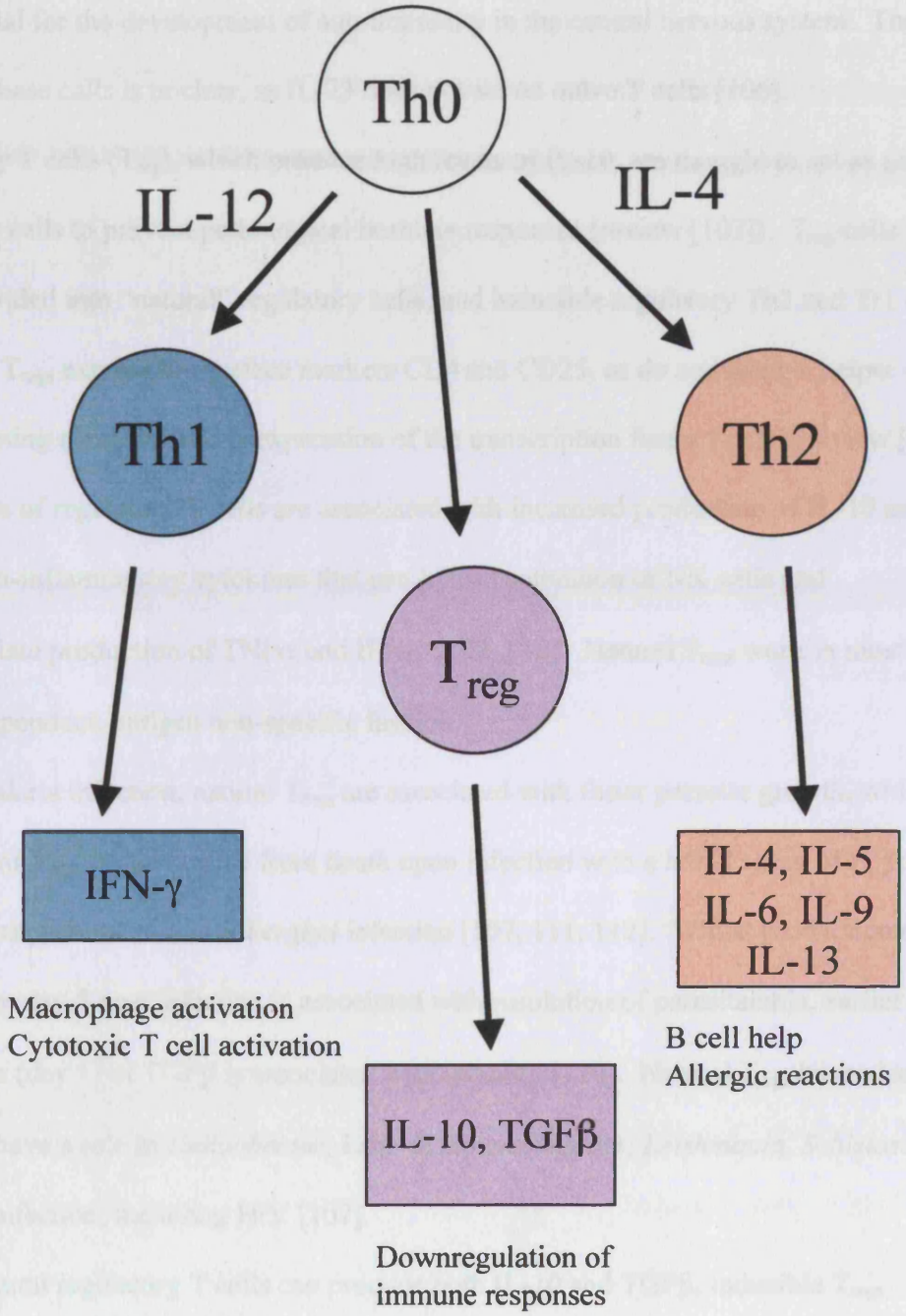


Figure 4. Development of Th1 and Th2 responses

induced by IL-23 [105]. These cells may be more closely related to Th1 than Th2 cells, as they express low levels of IFNγ, however IL-17 producing cells are highly pathogenic and are essential for the development of autoimmunity in the central nervous system. The origin of these cells is unclear, as IL-23 does not act on naïve T cells [106].

Regulatory T cells (T_{reg}), which produce high levels of IL-10, are thought to act as negative regulatory cells to prevent pathological immune responses (review [107]). T_{reg} cells can be further divided into "natural" regulatory cells, and inducible regulatory Th3 and Tr1 cells. "Natural" T_{regs} express the surface markers CD4 and CD25, as do activated T helper cells. Their defining characteristic is expression of the transcription factor FoxP3 (review [108]). The effects of regulatory T cells are associated with increased production of IL-10 and TGFβ, anti-inflammatory cytokines that can inhibit activation of NK cells and downregulate production of TNFα and IFN-γ [109, 110]. Natural T_{regs} work in mostly non-contact dependent, antigen non-specific fashion.

During malaria infection, natural T_{regs} are associated with faster parasite growth, while depletion of T_{regs} protects mice from death upon infection with a lethal strain of *P. yoelii*, and reduces parasitaemia in *P.berghei* infection [107, 111, 112]. Whilst production of TGFβ from day 5 post infection is associated with resolution of parasitaemia, earlier production (day 1) of TGFβ is associated with lethality [110]. Natural T_{regs} have also been shown to have a role in *Helicobacter*, *Listeria monocytogenes*, *Leishmania*, *Schistosoma* and viral infection, including HIV [107].

Whilst natural regulatory T cells can produce both IL-10 and TGFβ, inducible T_{regs} primarily produce either IL-10 (Tr1) or TGFβ (Th3) [113]. Tr1 cells may be again subdivided into those that produce only IL-10 and those that additionally produce Th1 and

Th2 cytokines (IFNγ and IL-5), though the significance of such a division is unclear [114]. Tr1 cells act in an antigen-specific fashion and are thought to function in the control of auto-immunity [115]. Th3 cells are induced in response to antigens in the digestive tract, and function in a mostly antigen non-specific fashion [115]. Different subsets of regulatory T cells have been suggested to be induced by different dendritic cell subsets [89]. $CD8^+$ T_{reg} ($CD28^+$ and $CD28^-$ subsets), $CD4^+CD25^-$ T_{reg}, NK T_{reg} and B_{reg} populations have also been described, however little work has been done on these as yet [115, 116].

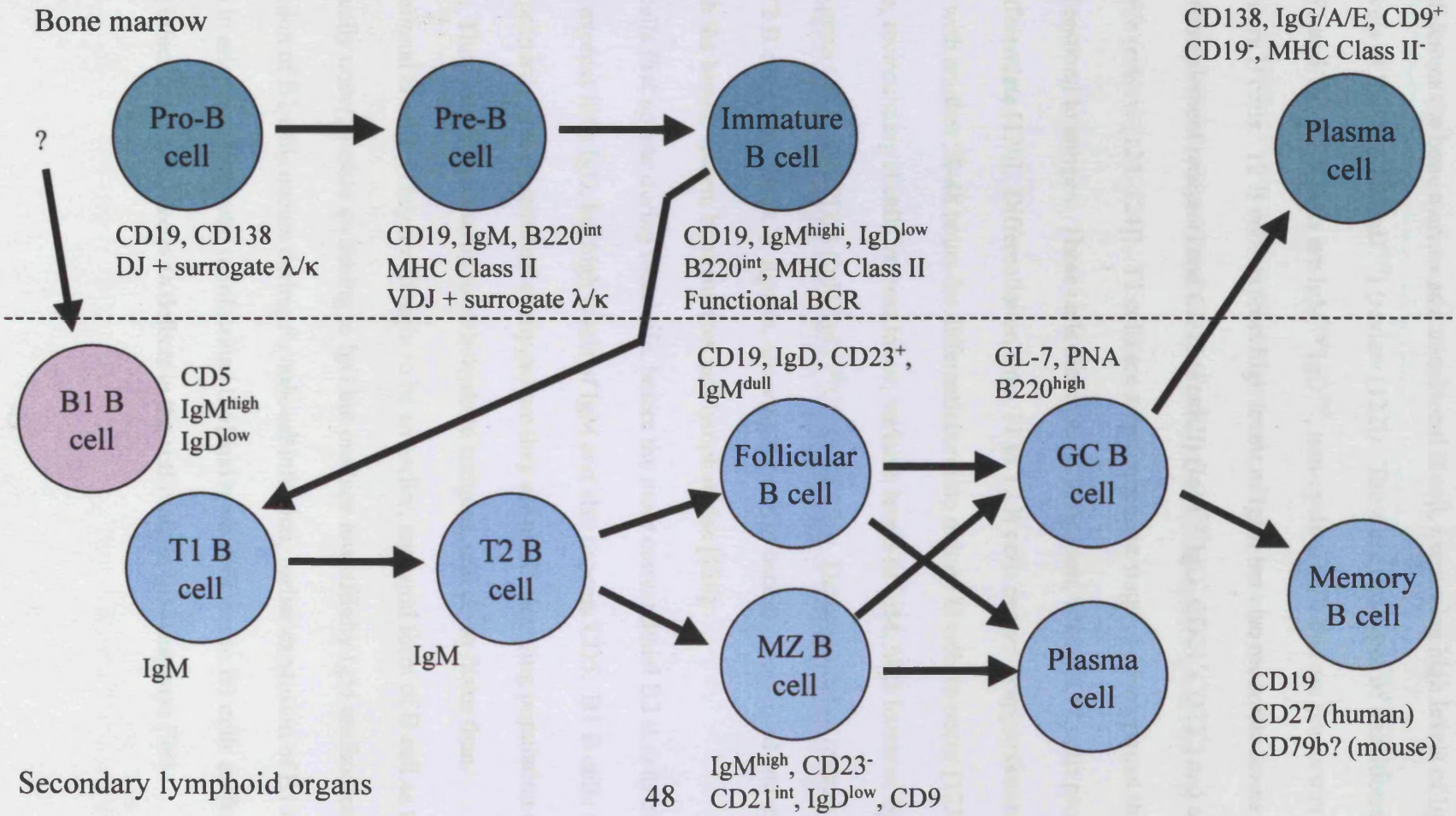
B cells

The major function of B cells is antibody production. B cells develop in several discrete stages in the bone marrow, the first of which is a progenitor B cell (pro-B cell). B cell developmental stages can be defined by surface markers, or by the state of re-arrangement of their immunoglobulin genes [117-119] (Figure 5). Antibodies are made up of light chains and heavy chains. Antibody genes are made up of constant (C), variable (V), diversity (D) and joining (J) chain segments. The V, D and J chain segments must be correctly rearranged and expressed on the cell surface with the C chain segments during B cell development in order for viable B cells to be exported from the bone marrow (review [120]). Heavy chain DJ rearrangment occurs in pro-B cells, and a surrogate light chain is expressed at this stage. Once DJ rearrangement is complete, heavy chain V genes are rearranged to join the D and J chains. Once an in-frame rearragement of the heavy chain has occurred, this is expressed on the cell surface together with the surrogate light chain as the pre-B cell receptor, and the cell becomes a pre-B cell (review [121]). Pre-B cells undergo a number of proliferation cycles, then light chain rearrangment can occur and, as the cell becomes an immature B cell, IgM is expressed on the cell surface.

Figure 5. Diagrammatic representation of the stages of B cell development

B cells develop in the bone marrow from pro-B cells to pre-B cells to immature B cells, which are released into the circulation. Immature B cells develop from transitional type 1 (T1) B cells to transitional type 2 B cells (T2) in secondary lymphoid organs. T2 B cells develop into either marginal zone B cells or follicular B cells, either of which can form extra-follicular plasma cells or germinal centres. Germinal centre B cells can differentiate into memory B cells or long-lived plasma cells. Most long-lived plasma cells are found in the bone marrow, however some can also be found in the gut and the spleen.

B1 B cells reside in the peritoneal cavity. It is not known at what developmental stage these cells separate from the rest of the B cells (B2 B cells)


Bone marrow
?
Pro-B cell
Pre-B cell
Immature B cell
Plasma cell
CD138, IgG/A/E, CD9+
CD19-, MHC Class II-
CD19, CD138
DJ + surrogate λ/κ
CD19, IgM, B220int
MHC Class II
VDJ + surrogate λ/κ
CD19, IgMhighi, IgDlow
B220int, MHC Class II
Functional BCR
B1 B cell
CD5
IgMhigh
IgDlow
CD19, IgD, CD23+,
IgMdull
GL-7, PNA
B220high
Follicular B cell
GC B cell
T1 B cell
T2 B cell
MZ B cell
Plasma cell
Memory B cell
IgM
IgM
IgMhigh, CD23-
CD21int, IgDlow, CD9
CD19
CD27 (human)
CD79b? (mouse)
Secondary lymphoid organs

The cell leaves the bone marrow as a transitional B cell, expressing high levels of IgM and low levels of IgD ($IgM^{high}IgD^{dull}$) (review [122]). There are two types of transitional B cells, T1 and T2. T1 B cells are $IgM^{high}IgD^{dull}$, non-cycling cells that are precursors of T2 and mature B cells. T2 B cells express high levels of IgM, but also moderate levels of IgD, CD21 (complement receptor) and CD23 (FcεRII) ($IgM^{high}IgD^{+}CD21^{+}CD23^{+}$) and are in cell cycle (review [123, 124]). T1 cells are an intermediate stage of development that cannot respond to antigen. These cells die upon BCR ligation, whereas T2 cells proliferate and differentiate [120]. Differentiation from T1 to T2 B cells occurs in approximately 48 hours, with another 24-48 hours for differentiation into mature B cells to occur [123]. Mature, recirculating B cells express lower, variable levels of IgM, high levels of IgD and also express CD21 and CD23 ($IgM^{+}IgD^{high}CD21^{+}CD23^{+}$). Development of mature B cells from T2 B cells requires BCR signals, allowing the cell to become long-lived and circulate through the blood, spleen, bone marrow and lymph nodes [124].

B1 B cells first appear during foetal life, before the more conventional B2 B cells can be found, express little IgD, but high levels of IgM and also express CD5. B1 B cells are found primarily in the peritoneal cavity, where they are a self-renewing population (review [125]). They respond primarily to carbohydrate antigens, and do so faster than conventional B cells. They are thought to be an earlier, ancestral form of B cell as they do not usually undergo class switching to IgG but produce low-affinity IgM antibodies. Expansion of B1 cells occurs during *P.chabaudi* infection, earlier expansion of B1 cells occurs in mice more resistant to infection [126] and mice deficient in B1 cells suffer more severe infections, associated with defects in the anti-malaria IgM response [99].

Marginal zone (MZ) B cells are a different line of development, thought to differentiate from conventional B cells between the T1 and T2 B cell stages [57, 127]. They express high levels of IgM but little IgD, express moderate levels of CD21 but low levels of CD23 (IgM^{high} $IgD^{dull/-}$ $CD21^{+}CD23^{lo}$) [128, 129]. They share many characteristics of B1 cells in that they can also respond to carbohydrate antigens, have a lower threshold for activation than conventional B cells and as such are important for responses for T cell-independent type II antigens [130, 131]. CD21/35 is the complement receptor CR1/2, and is expressed at higher levels on MZ B cells than on follicular B cells [129, 132]. Through complement (C3 & CR1/2), polysaccharide antigens are specifically targeted to MZ B cells [131]. As well as their involvement in responses to T cell-independent antigens, MZ B cells can also participate in responses to T cell dependent antigens. Upon activation they migrate into the B cell follicle, can present antigens to $CD4^{+}$ T cells, and are fully capable of forming germinal centres, undergoing somatic hypermutation and antibody isotype switching (review [129]) [133].

There are two methods of B cell activation, thymus dependent (TD) and thymus independent (TI) responses. TI responses can be further divided into two types (TI-I and TI-II). TI-I antigens include the bacterial cell-wall component lipopolysaccharide (LPS). TI-I antigens are mitogens that can activate B cells through receptors not including the specific B cell receptor (e.g. TLRs) [43]. Both TI-I and TI-II antigens can also generate memory B cells and secondary antibody responses [134, 135], although TI-II memory B cells may have a slightly different phenotype to TI-I and T-D antigen induced memory B cells [135]. TI-II antigens are multivalent antigens that activate B cells by cross-linking B cell receptors (BCRs) [136], however they activate only mature B cells and inactivate

immature B cells [43]. TI responses are also not completely T-cell independent, as T cells or cytokines such as IFNγ are still required for efficient proliferation and antibody class-switching [137, 138].

Most infectious agents include TD and TI antigens, with the majority of immune responses being to TD antigens. T cell help is mostly MHC restricted (i.e. cognate - the helping T cell must be specific for the same antigen presented by the helped B cell) and mediated by effector T cells. It requires both cell-contact, the interaction of CD40 and ICOS ligand (ICOS-L) on B cells with CD40 ligand (CD40L) and ICOS on T cells, and cytokine production (e.g. IL-4 & IL-21) (review [136]) [43, 139]. In certain conditions, however, T cell help may be non-cognate or bystander without involvement of the B cell receptor [139, 140]. Although germinal centres may form in the absence of cognate help, T cell help is required for the exit of cells from germinal centres and prevention of apoptosis [141].

TD antigens bind to the B cell receptor (BCR), which is part of a complex of molecules including CD19, CD21 and CD81. Any signal received through the BCR is modified and amplified by the co-receptor complex, and the co-receptor complex is required for correct formation and progression of the germinal centre reaction [142] (review [143]). Antigen binding leads to increased expression of MHC Class II and T cell co-stimulatory molecules on the B cell surface. The T cell receives signals from the MHC Class II and co-stimulatory molecules on the B cell and expresses its own co-stimulatory molecules. Either BCR or co-stimulatory signals from activated T cells can activate B cells to proliferate, and the BCR can bind either membrane-bound or soluble antigen [144, 145]. T cell derived cytokines (e.g. IL-4, IL-6), however, are also required for full B cell activation and germinal centre formation [144]. Germinal centre formation is reduced, although not

completely abrogated, in IL-4$^{-/-}$ mice [146], and paradoxically enhanced in a secondary response in IL-4$^{-/-}$ mice [147], possibly indicating more of a regulatory role, rather than an essential requirement, for IL-4 in germinal centre development.

Activated B cells proliferate then follow one of two further developmental pathways. Some move straight to antibody production, producing low-affinity IgM antibodies (i.e. extrafollicular plasma cells), but the majority of these cells are short-lived [148, 149] (review [150]). Whilst both early antibody-producing cells and germinal centre cells may be derived from the same proliferating cell, most activated B cells enter the germinal centre pathway of development [151].

Initiation of immune responses

Primary T and B cell responses are not initiated at the site where the antigen enters the body. Instead dendritic cells, as part of their activation process, migrate away from the site of antigen encounter to the secondary lymphoid organs, the draining lymph nodes and spleen [152] (review [55]). Here they can interact with antigen-specific T cells and B cells, causing their activation, proliferation and differentiation into effector cells. Effector T cells migrate out of the secondary lymphoid organs, towards the site of antigen encounter. Effector B cells (i.e. plasma cells) produce antibodies that circulate through the blood stream. Plasma cells also migrate to the bone marrow, which has a larger capacity for supporting long-lived plasma cells than the spleen. Migration is controlled primarily by upregulating and downregulating receptors for certain signalling molecules, thereby altering the cell's responsiveness to tissue-specific signals (review [153]).

Chemokines

Chemokines are small, 8-14 kDa secreted proteins that are involved in cell trafficking and migration. Four subtypes of chemokines have been identified, based on the number and spacing of cysteine residues – C, CC, CXC and CX_3C, CC and CXC being the major ones (review [154]). They play a crucial role in the formation of secondary lymphoid organs during ontogeny, and in the migration of all lymphocytes throughout the body. Chemokines act by attaching to the extracellular matrix and forming signal gradients that allow several different cell types to respond to the same signal. The chemokines CXCL13 (binds CXCR5), CCL19 and CCL21 (bind CCR7) are important for formation and maintenance of the splenic microarchitecture (review [155]). Chemokines are also important in ongoing immune responses, e.g. controlling the migration of dendritic cells into secondary lymphoid organs and the organisation of germinal centres [56, 156-159].

Many different cell types can produce chemokines, including dendritic cells and stromal cells. Some chemokines are expressed constitutively, others can be induced in an immune response to allow cells to infiltrate inflammatory sites. Alterations in the expression of chemokine receptors allow cells to home to specific sites at different stages of development. For example, cutaneous memory T cells express CCR4 for homing to the skin, whilst plasma cells express CXCR4 for homing to the bone marrow (review [154]) [160]. At least 40 chemokines have now been described, although not all of their receptors and mouse/human homologues have been identified (review [161]). The role of chemokines in migration is the best characterised, however other roles for chemokines are beginning to be elucidated, for example chemokine receptors have been suggested to act as PRRs in co-operation with TLRs (review [45]). Their receptors have also been shown to be

crucial in infection with HIV, virus tropism being determined by chemokine receptor expression (review [162]).

Tumour necrosis factor α and lymphotoxin

The cytokines tumour necrosis factor α (TNFα), lymphotoxin α (LTα) and lymphotoxin β (LTβ) are crucial for the correct formation of the splenic microarchitecture [163]. The relative importance of each cytokine in the formation of splenic architecture is difficult to decipher. Splenic formation still occurs in the absence of all three of these cytokines, indicating that they are not required for splenic ontogeny, however the microarchitecture of the spleen is radically altered in mice lacking all three cytokines, and to a lesser extent in mice lacking only one of these cytokines [164].

The most common form of TNFα is the secreted form, however it also exists in a membrane bound form anchored to the cell membrane by a hydrophobic domain [165]. Lymphotoxin is a trimer, made up of a combination of subunits α and β. The cytokine referred to as LTα is the α_3 form which is soluble (review [166]). LTβ is in fact $LT\alpha_1\beta_2$, is membrane bound by the β subunit [165], and LIGHT is another, membrane bound, member of this cytokine family, whose role in the formation of the splenic microarchitecture is as yet unknown [121]. LIGHT may have a role in autoimmunity [167], allorecognition and tumour regression (review [168]). The lymphotoxin, TNFα and LIGHT molecules and receptor combinatons are illustrated in Figure 6, and their signalling pathways are shown in Figure 7.

TNFα, LTα and LTβ are each expressed by several different cell types. TNFα is primarily expressed by macrophages, whereas LTα and β are mainly produced by T cells, B cells and NK cells [165, 169]. This production may be constitutive, but is more often induced upon

Figure 6. Diagrammatic representation of the subunit combinations and the ligand-receptor combinations of TNFα, lymphotoxin and LIGHT.

LTα (■) and β (■) subunits combine to form $LT\alpha_3$, $LT\alpha_1\beta_2$, $LT\alpha_2\beta_1$ and possibly $LT\beta_3$ TNFα, lymphotoxin and LIGHT bind to the LTβ receptor (■), TNFRI (■), TNFRII (■) and HVEM (■)

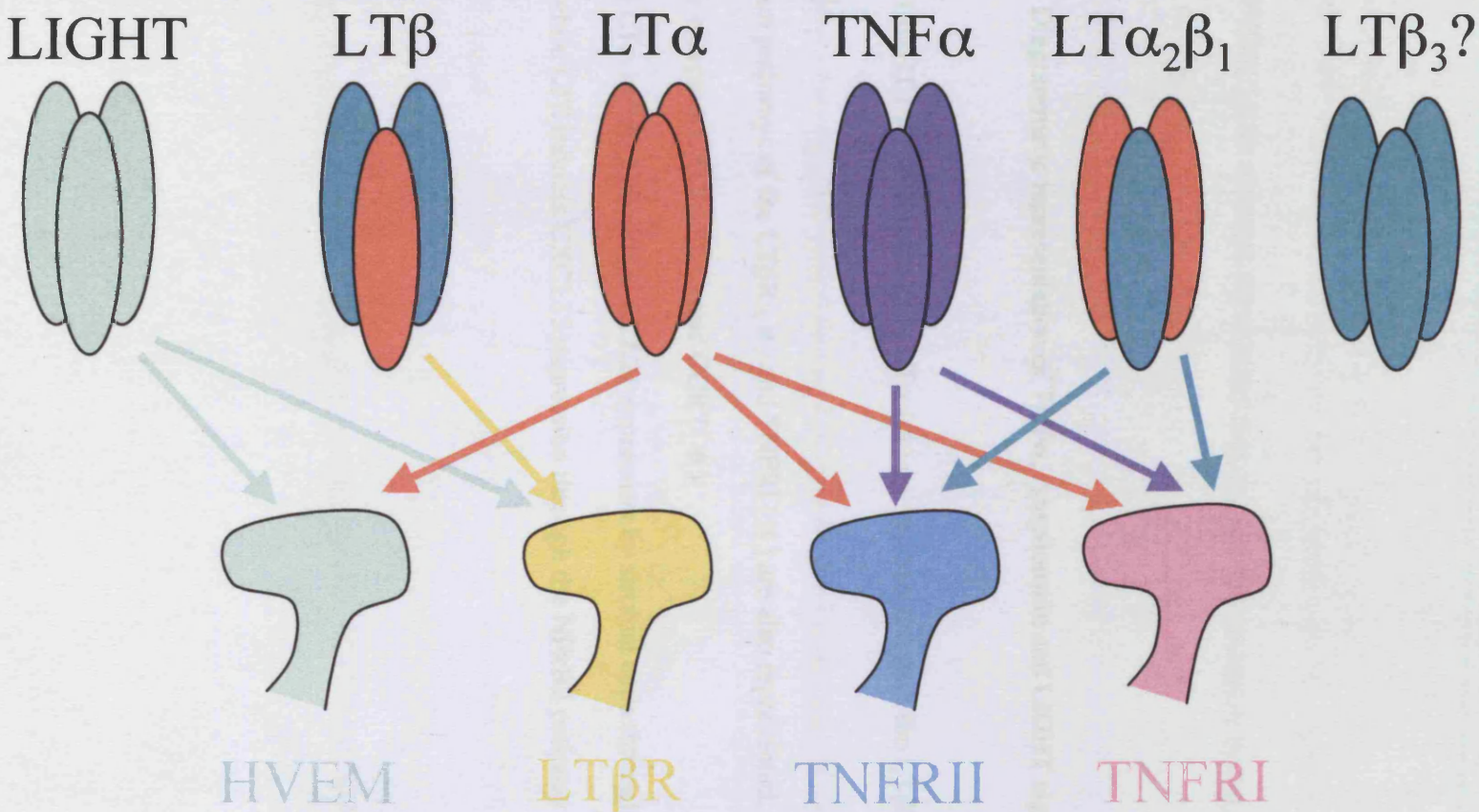
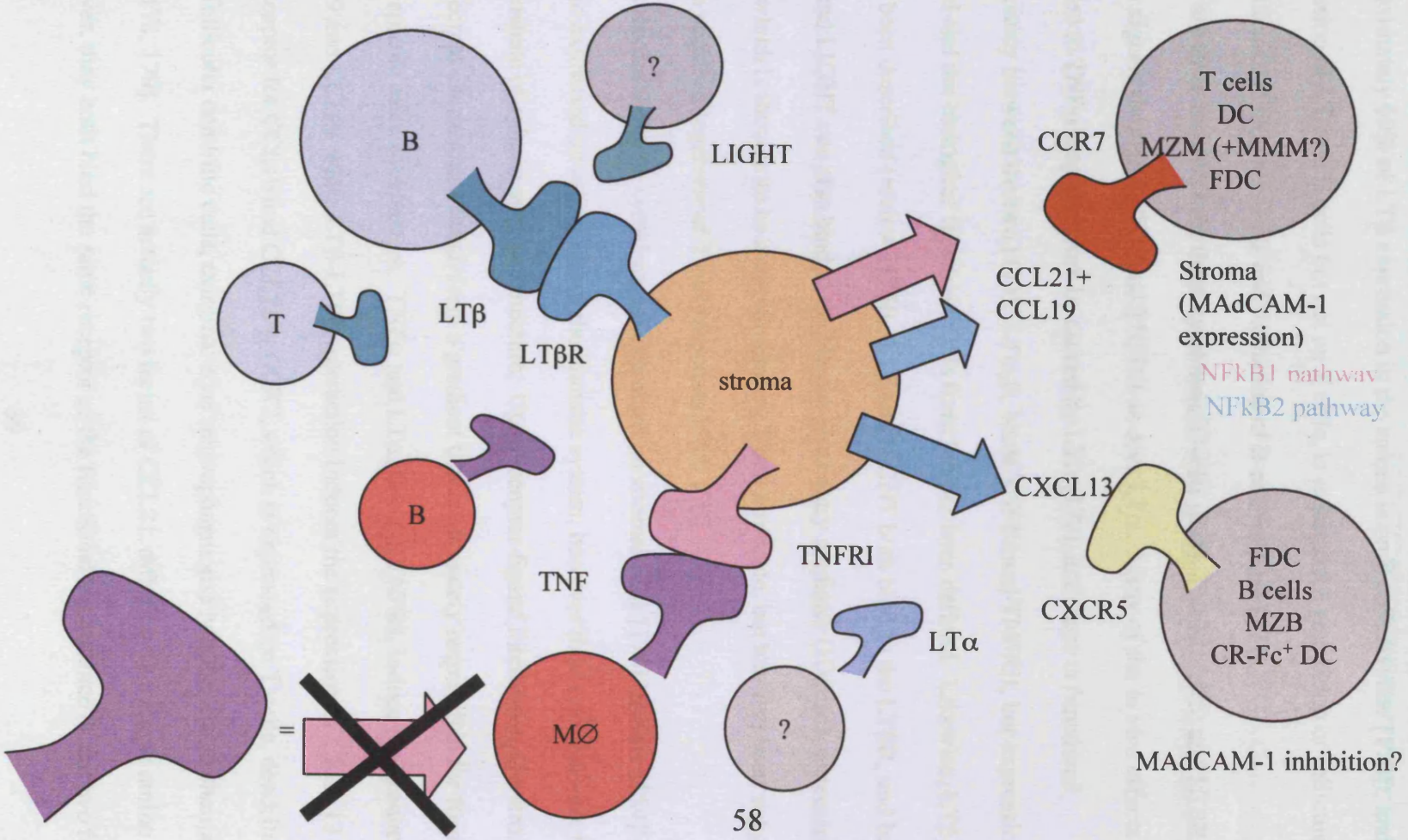

LIGHT
LTβ
LTα
TNFα
LTα2β1
LTβ3?
HVEM
LTβR
TNFRII
TNFRI

Figure 7. Diagrammatic representation of TNFα, lymphotoxin and LIGHT signalling pathways

LTβ (■), LIGHT (■), TNFα (■) and LTα (■) are represented with the LTβR (■) and TNFRI (■)

Downstream pathways of the LTβR (■) and TNFRI (■) are also represented, as are chemokine receptors CXCL13 (■) and CCR7 (■)

TNFα and LTα induce CCL19 and CCL21 expression by stromal cells through the NFκB1 pathway, while LTβ induces CXCL13 expression through the NFκB2 pathway


?
B
LIGHT
T cells
DC
MZM (+MMM?)
FDC
CCR7
T
LTβ
LTβR
stroma
CCL21+
CCL19
Stroma
(MAdCAM-1
expression)
NFkB1 pathway
NFkB2 pathway
B
CXCL13
TNFRI
TNF
FDC
B cells
MZB
CR-Fc+ DC
CXCR5
LTα
=
MØ
?
MAdCAM-1 inhibition?

activation by mitogens, anti-CD3 (T cells), antigen or IL-2 (T and NK cells) [165]. Approximately 60% of LTβ expression in the spleen is on B cells (review [170]), and expression of LTα on B cells but not on T cells, is required for generation of follicular dendritic cell (FDC) networks and formation of B cell follicles [171].

There are three receptors for these cytokines, TNFRI (p55), TNFRII (p75) and LTβR. TNFα signals through TNFRI and TNFRII as does LTα. Many of the *in vivo* effects ascribed to TNFα may therefore be caused by LTα [172], and there is functional redundancy between the two [173]. $LT\alpha_2\beta_1$ binds TNFRI and TNFRII, but expression is limited and the biological function of this form has not been defined. Likewise, $LT\beta_3$ has never been described (review [174]). LTβ and LIGHT both bind to the LTβR, and both LTα and LIGHT can also bind to the Herpes virus entry mediator (HVEM), expressed on T cells, which is thought to be a co- stimulatory T cell molecule, but has also been suggested to be a negative regulator of T cell responses [168, 175].

The receptors for these cytokines are expressed on stromal cells [176] (review [154]). They are also expressed on other cells of the immune system, however this is not relevant for maintenance of splenic microarchitecture. Upon receptor-ligand interaction, the stromal cells secrete chemokines which form a gradient that is ultimately responsible for formation of the splenic microarchitecture. TNFα and LTα, through TNFRI, induce the production of CCL19 and CCL21 while LTβ-LTβR interaction induces the expression of CXCL13 [177]. The receptor for CCL19 and CCL21 is CCR7, which is expressed on T cells, dendritic cells, follicular dendritic cells, marginal zone macrophages and lymphatic endothelium [154, 178, 179]. There are actually two forms of CCL21, differing by a single amino acid. However, they both bind the same receptor so the functional significance of the two forms

is unclear [161]. The receptor for CXCL13 is CXCR5, which is expressed on follicular dendritic cells, a subset of T cells, follicular B cells and marginal zone B cells [154, 155, 158].

Whilst the TNFRI and LTβR signalling pathways appear to act synergistically for formation of B cell follicles, T cells zones and FDC networks, at other times they act antagonistically. For example, LTβ signalling is required for the presence of the marginal zone, however in the absence of TNFRI or CXCR5 signalling the marginal zone is enlarged [180, 181]. Signalling through TNFRI is also required for the expression of MAdCAM-1 on the sinus endothelium [181], but T and B cell LTβ knockout mice have increased expression of MAdCAM-1 over the splenic white pulp [170].

Secondary lymphoid organs

Lymph nodes are made up of three concentric regions – the outer cortex, the central paracortex and the inner medulla (Figure 8A) [43]. The cortex contains the B cell follicles, while the T cells reside in the paracortex. The medulla consists of medullary cords and the medullary sinus, and contains mostly macrophages and plasma cells (review [163]). Lymph entering the lymph node drains into the marginal sinus at the edge of the lymph node, between the cortex and the capsule. It then filters through the lymph node and drains into the efferent lymphatic vessel. Blood enters through the lymphatic artery and drains through the lymphatic vein. Unlike the spleen, lymph nodes have a closed circulation in all species [182], therefore antigen can only enter via the afferent lymphatic vessels [43]. B cells and T cells may enter the lymph node by migration through high endothelial venules in the paracortex, next to the junction with the cortex. They then traffic through the paracortex and medulla, exiting through the efferent lymphatic vessel. Dendritic cells are

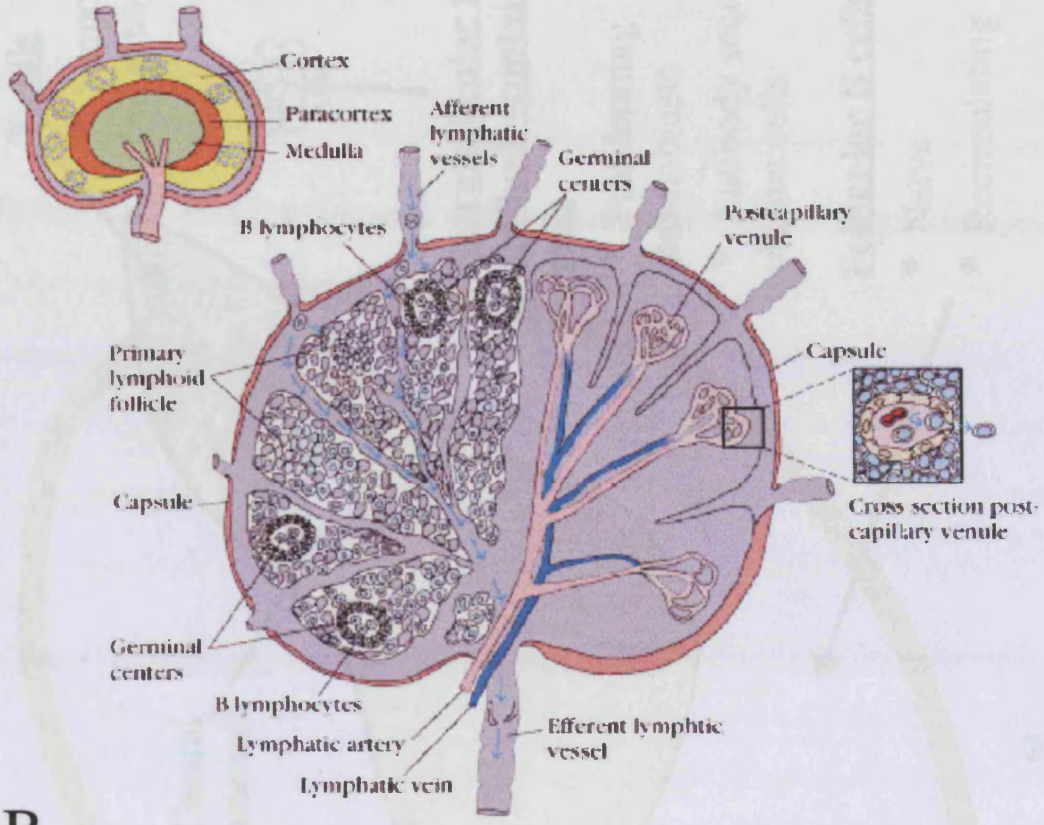
Figure 8. Structure of the spleen

A) Gross structure of lymph nodes showing cortex, paracortex and medulla. Reproduced from [43]

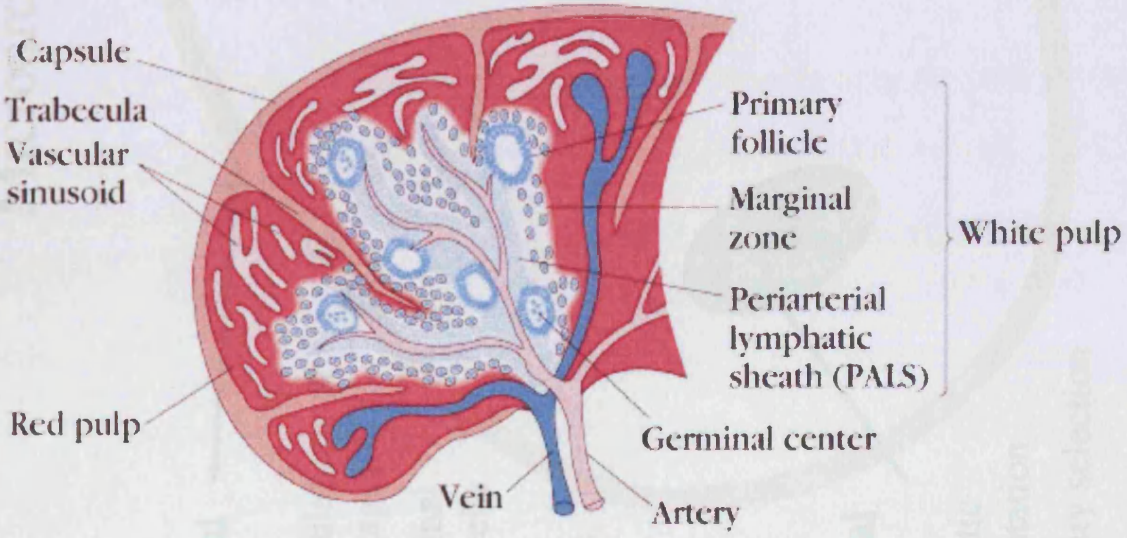
B) Gross structure of the human spleen showing red pulp and white pulp. Reproduced from [43]

C) Microarchitecture of the spleen showing B and T cell zones, germinal centres, marginal zone and extrafollicular foci of plasma cells

A

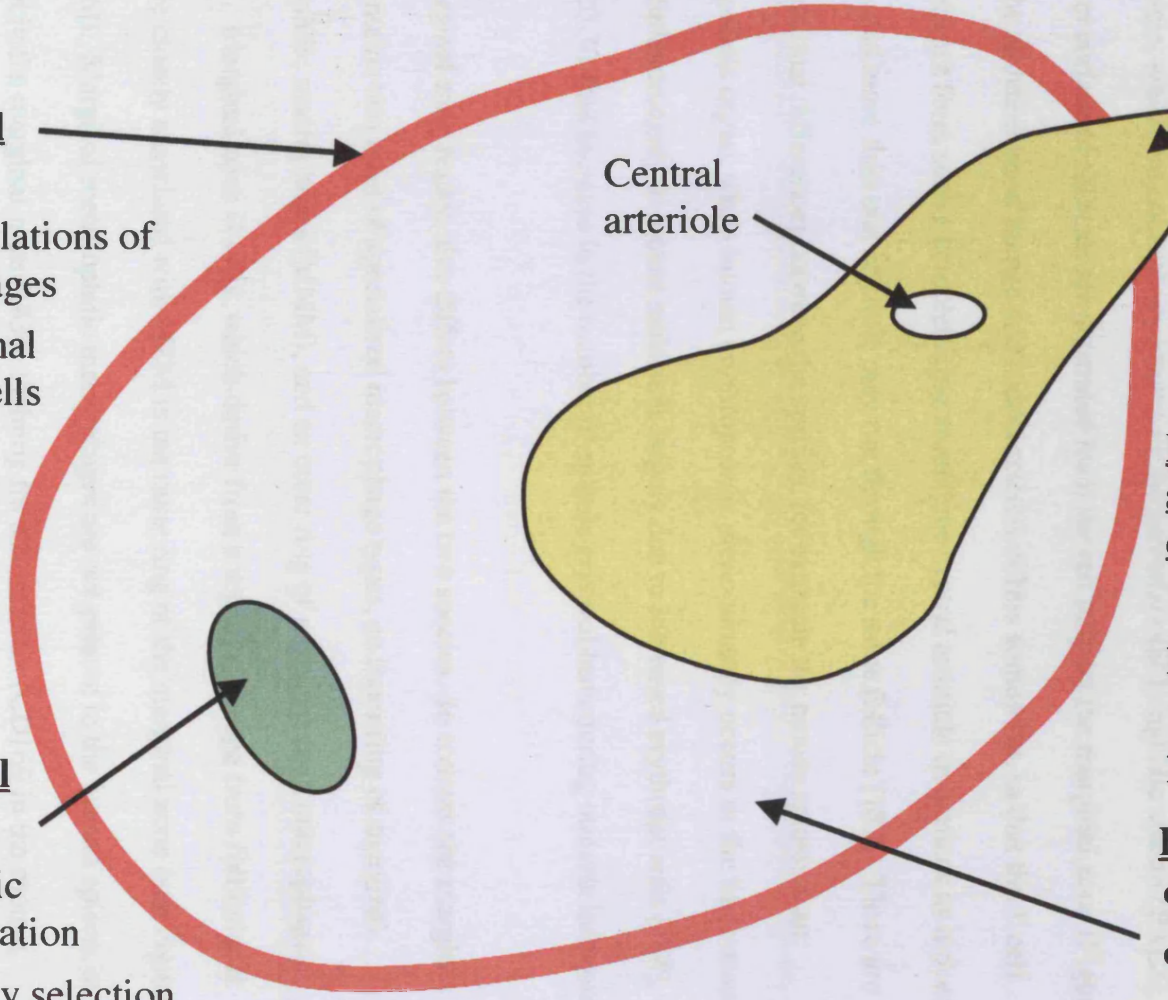

Cortex
Paracortex
Medulla
Afferent lymphatic vessels
Germinal centers
B lymphocytes
Postcapillary venule
Primary lymphoid follicle
Capsule
Capsule
Cross section post-capillary venule
Germinal centers
B lymphocytes
Lymphatic artery
Lymphatic vein
Efferent lymphtic vessel

B


Capsule
Trabecula
Vascular sinusoid
Red pulp
Vein
Artery
Germinal center
Primary follicle
Marginal zone
Periarterial lymphatic sheath (PALS)
White pulp

C

Microarchitecture of the spleen



Marginal zone

- 2 populations of macrophages
- Marginal zone B cells
- Some dendritic cells

Central arteriole

T cells

- Support B cell activation

Extrafollicular foci of antibody-containing cells

- Proliferating plasmablasts
- Antibody secreting plasma cells

Germinal centre

- Somatic hypermutation
- Affinity selection

Follicular B cells

- Naïve
- Recirculating

thought to enter through the lymphatic system, and transit through the paracortical cords for encounters with T cells (review [163]).

The spleen is a secondary lymphoid organ composed of two main regions, the red pulp and the white pulp, which can be readily distinguished (Figure 8B) [43]. The white pulp is centred around the central arteriole, and is divided into the inner T cell zone and the outer B cell follicle, which are collectively known as the periarteriolar lymphatic sheath (PALS). The outer surface of follicles are separated from the red pulp by the marginal zone (Figure 8C). The architecture of human and rodent spleens differs somewhat, in that the T cell zone does not form such a complete ring around the central arteriole in humans as it does in rodents, and more than one arteriole may run through the same follicle [183]. There are also functional differences between the species, for example the mouse spleen is an erythropoietic organ, while human erythropoiesis predominantly occurs in the bone marrow [183]. Splenomegaly in mouse malaria is largely due to increased erythropoiesis [184], with a 20-30-fold increase in the number of splenic erythroblasts during malaria infection [185].

The marginal zone region also differs between the two species. In rodents the marginal zone contains two rings of specialised macrophage types, an inner ring of marginal metallophilic macrophages (MMM), and an outer ring of marginal zone macrophages (MZM). Marginal zone B cells, which derive from a separate lineage from follicular B cells, are closely associated with MZM in the outer ring of the marginal zone (see Figure 29; [186]). Marginal metallophilic macrophages are not present in the human spleen, at least not in the marginal zone [183]. Staining for sialoadhesin (CD169 in the mouse,

expressed on MMM) however, picks up macrophages surrounding sheathed capillaries in the perifollicular zone.

The perifollicular zone is a region outside the marginal zone that is sometimes identified as part of the marginal zone, however it contains a high concentration of erythrocytes [183]. Neither the perifollicular zone nor the sheathed capillaries are present in rodent spleens. Interestingly, the centre section of the sheathed capillaries is surrounded by two layers of macrophages, the inner of which is sialoadhesin negative [183]. It is possible that the macrophage populations found in the marginal zone of the rodent spleen are the same cells surrounding the sheathed capillaries in the perifollicular zone of the human spleen, but this could only conclusively be tested by a functional study on these cells. It has been postulated that the perifollicular zone in humans serves much the same function as the marginal zone in rodents [183].

The exact path of blood circulation through the spleen is unclear. Both human and rodent spleens contain splenic cords and sinuses, however there are some structural differences which may affect circulation. There are areas where blood is contained within endothelium and areas where is is not in human and rodent, with a network of fibroblasts that may tend towards being contractile myofibroblasts that aid in circulation. Blood enters the spleen through the central arteriole, which in rodents opens out into the marginal zone and red pulp. The rodent spleen is described as having both "open" and "closed" circulation. The red pulp is made up of irregularly shaped blood vessels, the splenic sinuses, and the cell-filled spaces between them, the splenic cords, which form the "closed" and "open" portions of the splenic circulation respectively [183, 187].

The splenic cords are composed of a network of fibroblasts supporting B and T cells, erythrocytes, thrombocytes, granulocytes and plasma cells. The central arteriole opens into the splenic sinus, at the inner edge of the marginal zone. Here these cells can interact with the blood, which then drains into the splenic vein. Branches from the central arteriole carry blood through the marginal zone to the red pulp cords, and empty out into the cord spaces. Blood then drains back into the sinuses through slits in the sinus walls [183]. The arterial system in the human spleen is more elaborate than in the rodent spleen, and contains structures such as the sheathed capillaries which are entirely absent in rodents. Nevertheless, blood also opens out into the splenic cords and perifollicular zone, where it is no longer constrained within an endothelial layer but moves through a fibroblastic network to re-enter the splenic sinuses [183].

Splenic microarchitecture

B cell follicles are mostly made up of recirculating, mature B cells. On encounter with antigen germinal centres are formed within follicles, where B cells undergo somatic hypermutation and affinity selection (Figure 8C) [188]. Cells exiting the germinal centre reaction then differentiate into plasma cells, or memory B cells in the MZ. The marginal zone contains two populations of macrophages, marginal zone B cells and memory B cells. The marginal zone macrophages (MZM) are located on the outer rim of the MZ, closest to the red pulp, and interact with marginal zone B cells. The marginal metallophilic macrophages (MMM) are located on the inner edge of the MZ next to the white pulp. The sinus lining endothelial cells lie between the two populations [186]. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is expressed on the sinus-lining endothelial cells in the marginal zone [189]. The role of MZM and MMM in infection is thought to be mostly

phagocytosis of pathogens (review [190]), however they have been shown to produce IFNα in response to viral infection [191].

Follicular dendritic cells

Follicular dendritic cells (FDCs) are present within B cell follicles and have numerous dendrites that when associated with immune complexes are known as iccosomes. Little is known about these cells, which are not true dendritic cells, are not phagocytic and do not present antigens to naïve T cells (review [192]). Their origin is uncertain, they have not been thought to be of hematopoietic origin, as they are radio-resistant and have several cell surface molecules in common with fibroblasts (review [193]), however recent work has found donor-derived FDCs after adoptive transfer [194], and derived FDC-like cells from monocyte precursors [195]. They do not express MHC Class II molecules on their surface, although they do bind microvesicles containing MHC Class II and other membrane-bound proteins, and therefore cannot present antigens directly to T cells [196].

FDCs activate B cells, inducing proliferation and preventing apoptosis and are required, along with T cells, for germinal centre formation [197, 198]. They bind antigen-antibody complexes on their surface, which provide a basis for affinity maturation of germinal centre cells, and express other co-stimulatory molecules for B cell activation [199, 200].

Although the role of FDCs in the germinal centre reaction and the maintenance of memory has been questioned, mice lacking immune complexes on FDCs still have severely reduced antibody responses (review [192]) [201]. In addition to presenting antigen on immune complexes, FDCs also secrete cytokines such as IL-6 and IL-15, which induces antibody production and enhances terminal differentiation of B cells [192, 202]). Finally, FDCs can

retain antigen-antibody complexes on their surface for months, and may therefore be important in maintaining antigen-dependent memory cells (review [192]).

Germinal centres

Antigen entering the secondary lymphoid organs from the blood encounters B cells in the marginal zone. Upon activation, MZ B cells migrate into the B cell follicle and may transport antigen to FDCs [203]. Both MZ B cells and activated follicular B cells can interact with antigen-specific T cells to form either germinal centres (GCs) or plasma cells, although whether MZ B cells or follicular B cells do so faster is debatable [121, 204]. Germinal centres become visible in B cell follicles from day 4 after antigen exposure [151]. Germinal centres are formed from as few as 3 B cells, which undergo rapid proliferation, with a cell cycle time of approximately 6-7 hours (review [188]) [205]. They fill the centre of the B cell follicle within 3-4 days of antigen exposure, pushing the follicular B cells out to form a follicular mantle, then they divide into two zones, the dark zone and the light zone [205].

The dark zone contains the proliferating centroblasts, while the light zone contains the non-proliferating centrocytes that are undergoing selection on the basis of their affinity for antigen held on follicular dendritic cells (review [188]). Selection requires the centrocytes to be activated through the B cell receptor – i.e. to compete for antigen binding with antibody in immune complexes, eventually replacing this antibody with higher affinity antibody (review [206, 207]). Centrocytes may also internalise some of this antigen and present it on MHC Class II molecules in order to interact with Ag-specific T cells, as a requirement for memory B cell survival [208].

The classical model of germinal centre reactions is that cells undergo several cycles of centroblast division and mutation in the dark zone, followed by centrocyte selection in the light zone and differentiation into high affinity plasma cells or memory B cells. However, the spatial separation of these two processes in germinal centres of the spleen has been questioned. It has been suggested that the separation of centroblasts and centrocytes is temporal rather than spatial (review [209]). In any case, the dark and light zone structure is no longer seen by 3 weeks after antigen exposure [205], although germinal centres may still be visible up to 100 days after immunisation [210].

The processes that occur in the germinal centre; somatic hypermutation, affinity maturation and isotype class-switching, are well characterised. Somatic hypermutation is the mutation of antibody variable (V) regions in centroblasts in order to produce cells of differing affinity for antigen (review [211]). Whilst this mutation is specifically directed at the variable regions, and does not involve the constant regions, base-pair substitutions are random [208]. This allows for production of specific antibodies to any antigen, however it also necessitates the death of many centrocytes producing high affinity self-reactive antibodies (review [212, 213]). Somatic hypermutation can first be seen 8 days after immunisation and peaks at 14 days post-immunisation [151].

Affinity maturation is the selection and survival of those centrocytes that have greatest affinity for the antigen bound to follicular dendritic cells (review [214]). Although the germinal centre reaction is necessary for these processes to take place, some affinity maturation continues after the germinal centre reaction has finished [215]. Antibody affinity has been shown to remain high for up to 12 months after intra-muscular

immunisation, although affinity declines over the same time period after oral immunisation [216].

Antibody isotype switching from IgM to IgG, IgA or IgE commonly takes place during the germinal centre reaction, however it can also occur in extrafollicular foci of plasma cells [151]. Isotype switching often occurs with affinity maturation during the germinal centre reaction, however it is also possible for isotype switching to occur without affinity maturation and vice versa (review [217]). Different antibody isotypes are defined by their Fc portion, which enables them to perform different functions. For example IgA is produced mostly at mucosal surfaces, IgE is involved in allergic responses (review [218]), IgD is mostly found bound to the cell surface, where it functions as part of the B cell receptor (review [219]), and IgG is the only isotype to be secreted across the placenta (review [220]).

IgM is the first class of antibody to be produced during a primary immune response as it is the "default" isotype that is expressed by newly formed B cells. "Natural" antibodies, that are germline-encoded and are produced by a host that has never been exposed to antigen, are also of the IgM isotype. IgM is thought to be specifically involved in immune responses to bacterial infection, and may also be involved in immune responses to viruses [221].

IgG is the most abundant antibody isotype in the serum, approximately 80%, and in most cases is the isotype produced after antibody class switching. IgG can be divided into several subclasses – IgG1-4. IgG2 can be further subdivided into IgG2a and 2b in mice, while IgG4 exists in humans but not in mice [222].

The different subclasses each have different activities, e.g. human IgG3 is the most effective IgG subclass for activating complement, whilst IgG1 and IgG3 are the most effective at mediating opsonisation [43].

Human and mouse antibody isotypes differ in IgG subclasses, the functions of different subclasses do not exactly correspond. Human IgG2 is thought to be equivalent to mouse IgG3, and human IgG1 and IgG3 to mouse IgG2a and IgG2b [223]. Different cytokines have been implicated in the selection of different antibody isotypes, e.g IFN-γ is thought to be required for switching to IgG2a and IgG3 in mice [138, 224], IL-4 for switching to IgG1 [225], IL-4, IL-13 and CD40 for switching to IgE (review [218, 226]). Isotype switched germinal centre cells and plasma cells become visible from 6-8 days after immunisation [151, 227].

Plasma cells and memory B cells

Cells that survive the germinal centre reaction become either plasma cells or memory B cells. Some long-lived plasma cells are retained in the spleen, however the spleen has a finite capacity to support plasma cells [228]. The majority migrate to the bone marrow, which can support a larger number [229] (review [230]). In this location they may persist for life [231-233] (review [234]), although it has been suggested that in fact there is continual turnover of plasma cells maintaining the long-term antibody responses [235]. Memory B cells do not constitutively produce antibodies, although they are capable of producing high affinity, isotype switched, antibody much faster than naïve B cells upon restimulation with antigen. Memory B cells can also be found, together with MZ B cells, in the marginal zone of the spleen [236]. Human memory B cells may express CD27 [237], however it has not been shown to be expressed on memory B cells in mice. Instead, CD27

ligation may inhibit plasma cell development and induce memory B cell differentiation [238, 239], or accelerate germinal centre formation [240]. It has also been suggested that $CD27^+$ B cells in humans are not memory B cells but are in fact circulating MZ B cells [241]. Although MZ B cells do not recirculate in rodents [242], they have been suggested to do so in humans [183].

The study of memory B cells in mice therefore remains difficult. They can be identified by functional assays and antigen binding, or as non-GC, non-antibody producing somatically mutated B cells. Of these methods antigen binding, by ELISPOT or flow cytometry, provides the best way to look at memory B cells in large numbers (e.g. the memory B cell population of the spleen).

The mechanism of B cell memory persistence is still somewhat controversial (review [243-248]. A true memory response should remain after the clearance of antigen, however it has been hypothesised that B cell memory cannot persist without residual amounts of antigen. It has also been suggested that bystander activation of memory B cells may serve to maintain them, and specific plasma cells, in the absence of antigen [235]. Memory to smallpox vaccination has been used as a useful example of memory maintenance in humans without the possibility of re-exposure to specific antigen. While this does not rule out non-specific polyclonal stimulation as a mechanism for the maintenance of memory, the data does demonstrate a stable pool of memory B cells and long-lived plasma cells persisting for up to 75 years [249, 250]. Antibody-secreting B cells have been shown to be important for the maintenance of $CD4^+$ T cell memory [251], however the importance of T cells in the maintenance of B cell memory is not known. $CD8^+$ T cell memory maintenance appears to be independent of persisting antigen (review [243]).

Immune responses to primary malaria infection

The immune response to experimental malaria infection in animal models indicates a role for both T and B cells, with $CD4^+$ and $CD8^+$ T cells critical for the immune response to liver stages of the parasite [96, 252, 253], whilst $CD4^+$ T cells and B cells are critical for immune responses to blood stages [254-257].

$CD4^+$ T cells are required for clearance of the acute erythrocytic infection, with CD4 knockout mice or mice depleted of $CD4^+$ T cells being unable to control the acute infection [100, 254, 258, 259] (review [260]). Depletion of $CD4^+$ T cells from immune mice renders them fully susceptible to re-infection [258]. Transfer of either naïve or immune $CD4^+$ T cells allows T cell deficient *nu/nu* mice to clear an otherwise lethal *P.berghei*, *P.c.adami* or *P.c.chabaudi* malaria infection, with transfer of immune cells clearing parasitaemia ealier than transfer of naïve cells [255, 256, 261]. The relative importance of different elements of the immune system depends, however, on the parasite species, as transfer of $CD4^+$ T cells from immune to naïve mice does not protect the recipient against *P.vinckei* infection [258], but does protect against *P.berghei* infection [256]. Transfer of immune T cells alone into combined T and B cell deficient SCID or $Rag^{-/-}$ mice protects them from death, but does not allow them to clear a *P.c.chabaudi* infection [255, 262]. Transfer of both B and T cells is protective however, and this protection is increased if B cells are activated with malaria antigens before transfer [255, 262]. T cells alone can therefore protect against death from infection, although they cannot produce sterile immunity without B cells and preferably antigen as well.

Other cell types that have been shown to have a role in immune response to malaria include γδ T cells, NK cells and NK T cells. γδ T cells contribute to the control of both acute [263]

and chronic parasitaemia (review [264]) [259, 265]. NK cells are activated by malaria infection [266], and their role in immunity to malaria is both production of IFNγ [267] and cytotoxicity [268] (review [269]). NK T cells have a role in both the acute and chronic phases of malaria infection, produce high levels of both IFNγ and IL-4, and may enhance the B cell and antibody response [249, 270-272].

There are two phases of the immune response to erythrocytic *P.chabaudi* infection. The initial phase, during the first 14 days, is characterised by high levels of cytokines such as IFNγ, early production of which is critical for control of acute parasitaemia [273, 274] and protection against cerebral malaria [275]. These cytokines are produced by Th1-type $CD4^+$ T cells, and also by cells of the innate immune system, such as γδ T cells and NK cells [265, 267, 276-278] (review [279]). The second phase of the response, during the low level chronic parasitaemia, is a Th2 type response with low levels of IFNγ production in *P.chabaudi*, *P.cynomolgi* and *P.knowlesi* infection [28, 104, 280] that provides very effective help for malaria-specific antibody production. There are conflicting reports on the timing and importance of a Th2 response in *P.falciparum* infection in humans [281, 282], whilst in *P.vinckei* infection in mice, the Th2 response appears to be less important that in *P.chabaudi* infection [283]. In *P.berghei* infection, a low Th2 response with decreased IL-4 and TGFβ and increased IFNγ in suceptible mice compared to resistant mice, has been associated with development of cerebral malaria [284].

The trigger for this Th1 to Th2 switch is unknown, although it may be induced by B cells or antibody as B-cell deficient mice maintain a Th1 type response [285-287]. It does not, however, require the production of IL-4 [288], which can be secreted by Th2-type $CD4^+$ T cells, as well as non-B, non-T cells that increase dramatically in number in the spleen

between days 9 and 23 post-infection [289]. It has been suggested that the trigger for IL-4 production by these cells is stimulation with IL-3 from an unknown source, and/or cross linking of Fcγ receptors [289]. The importance of IL-5 and IL-13 in malaria infection has not been determined.

Pathology of malaria infection

Malaria disease is associated with fever (in humans), hypothermia (in mice), anaemia and cerebral pathology. Whilst some of these symptoms may be caused in part by the parasite (e.g. schizogony, sequestration), most are thought to be due to excessive responses by the host. The cytokine TNFα is critical for protective immune responses in the acute infection [151], however too much TNFα has been associated with increased pathology and cerebral malaria in particular in human infections [290]. In rodent malaria infection, elevated production of TNFα occurs in mice susceptible to *P.berghei* (ANKA) cerebral malaria [284], but also in mice resistant to *P.berghei* (ANKA) cerebral malaria [291]. In *P.yoelii* infection, increased levels of TNFα were found in lethal infection compared to non-lethal infection, however $TNF\alpha^{-/-}$ mice were not protected from death [292]. TNFα induced upregulation of the adhesion molecule ICAM-1 has been suggested to correlate better with the development of cerebral malaria [292]. Earlier work, however, did not differentiate between the cytokines TNFα and LTα, and many of the effects originally attributed to TNFα are now thought to be caused by LTα [172].

The anti-inflammatory cytokines IL-10 and TGFβ downregulate the production of TNFα and thereby ameliorate pathology in *P.chabaudi* infection [110, 279, 293, 294]. In human malaria infections, reduced levels of IL-12 and TGFβ are associated with severe disease [295, 296], however TGFβ has also been associated with the pathogenesis of cerebral

malaria [297, 298], possibly through a synergistic mechanism with TNFα [297]. Low levels of IL-10 in *P.falciparum* infections, particularly a low IL-10:TNFα ratio, have been associated with severe malarial anaemia [130, 299].

Production of an appropriate immune response to malaria is therefore thought to involve a fine balance between pro and anti-inflammatory responses. Indeed, there is evidence that the ratio of pro to anti-inflammatory cytokines may be more important than the absolute level of those cytokines (review [300-302]).

B cells and antibody responses to malaria infection

Antibody responses to malaria antigens in humans are detectable after the first infection, and are associated with resistance to clinical disease [128, 184, 293, 303-312] (review [262]). However many anti-malaria antibodies do not last long after the end of the transmission season and are unable to protect against re-infection with *P.falciparum* [177, 306, 311, 313, 314]. *In vitro*, antibodies to specific *P.falciparum* antigens have been shown to prevent parasite invasion [306, 315, 316].

B cells are more important for final clearance of blood-stage *P.chabaudi* parasitaemia than in the control of acute parasitaemia, as μMT mice lacking B cells can control the acute infection but develop a chronic, persistant infection that remains for life [287, 317, 318]. J_HD mice, however, which are also deficient in B cells, were able to control the acute parasitaemia in *P.chabaudi* and *P.vinckei* infection, but suffered a lethal infection with *P.yoelii* [319], indicating that B cells are more important in infection with *P.yoelii* than in infections with other malaria species. B cells and antibody responses are continually dependent on T cells, however, as transfer of immune B cells to T cell deficient mice could not protect them against death from secondary malaria infection [261].

The importance of B cells in immunity to malaria is due to their production of antibodies. In human infections, and in infections of monkey with *P.falciparum*, transfer of serum antibodies can reduce parasitaemia in a stage-specific manner [320, 321] (review [262, 322, 323]). Protection against human malaria infection has been associated with antibodies of the IgG1 and IgG3 isotypes [324, 325]. Although antibodies can prevent invasion of red blood cells by parasites; their main effect seems to be antibody-dependent cellular inhibition (ADCI) of invasion in co-operation with blood monocytes [326], with antibodies binding FcγRII on monocytes, causing the release of soluble factors responsible for parasite killing [213]. Antibodies may also be responsible for parasite clearance in an Fc receptor independent mechanism [327]. In mice, transfer of immune serum can protect against challenge infection from *P. yoelii* but not *P. vinckei* [99, 258], effectivity of the serum increasing with the number of infections of the donor. This protection has been shown to be mediated by IgG, not IgM [17], and is associated with IgG1, IgG2a, IgG2b and IgG3 isotypes [327-329].

Despite the importance of antibodies in immunity to malaria, very little is known about the malaria B cell response in humans or mice (review [262]). Inappropriate antibody responses may contribute to disease, as elevated IgE levels have been associated with cerebral malaria [330]. Whilst some vaccination strategies have been successful in mice [328, 331-334], transfer of these strategies to clinical application has proved problematic [306, 335-338] (review [339-341]).

The spleen in immune responses to malaria

The spleen has three distinct roles during malaria infection. It is a site of haematopoisis and erythropoiesis, particularly in the mouse [184, 342, 343], it is a major site for the

removal of pRBC from the circulation [306, 344] and it is a site for the generation of immune responses (review [172]). The spleen increases several fold in size during acute malaria infection [184, 345], and undergoes extensive, yet temporary, alterations in its microarchitecture [345-348]. The significance of these alterations is unclear, and has been hypothesised to be a protective mechanism to prevent infection of the erythropoietic beds of the spleen [347]. Despite these alterations, clearance of the parasite and development of antibody responses still occurs [346]. The trigger for these alterations in the splenic microarchitecture is also unknown. It has been suggested to be an over-reaction of the host immune response (review [172]), however it may also be caused by parasite antigens activating TLRs, as TLR4 activation by LPS can induce some of the same changes that are seen in the immune response to malaria [349] and parasite antigens have been shown to stimulate TLRs [350]. Replacement of the spleen by a splenic cell suspension has the same effect as splenectomy and increases susceptibility to malaria infection, indicating a role for splenic stromal cells in the immune response [258, 351].

Immunity to re-infection

Malaria has been studied for many years, however we still know relatively little about how the body reacts to it. There are different views of what effective immunity to malaria consists of (immunity to parasitaemia or immunity to symptoms of disease), and why it takes so long to develop, which are not mutually exclusive (review [352]). The first view is that much of the pathology caused by malaria infection is due to an over-reaction of the immune response (review [353]) or an inappropriate immune response [110]. Immunity to disease is acquired slowly and in stages, the first being resistance to the most severe forms of disease, then over time a lessening of other symptoms (review [354]). This would

suggest that "immunity to malaria" is perhaps not sterile immunity to infection, but only the body learning how to produce an appropriate response, the memory of which is short-lived. Even after many years of living in an endemic area, resistance to disease can be lost by moving to a non-malarious area for approximately 12 months [18].

The second view is that immunity is very strain-specific, and that only after infection with many different strains is a sufficient memory repertoire built up to protect against most infections. Indeed, analysis of strains infecting children showed that some strains were found only in younger children, presumably the prevalent strains in that area, and that patients did not have antibodies to the strain they were currently infected with ([355-357]). Specific, more prevalent, isolates were also associated with more severe malaria, indicating that immunity to severe disease may be the immunity to those isolates which cause severe disease [358, 359]. This would suggest that cross-isotype immune responses would be beneficial to the host, however it has been suggested that cross-reactive immune responses would be weaker and more likely to cause chronic infection [360]. It has also been suggested that immunity to malaria may be associated with the ability to maintain chronic, asymptomatic infections [361]. Such contradictions emphasise that acquisition of immunity to malaria is a complex process, thus only by fully understanding the interaction between the parasite and the immune system, and what constitutes an effective immune response, can we hope to eliminate this disease.

It has been hypothesised that the malaria parasite has the ability to downmodulate the immune response, suppressing the ability of dendritic cells to present antigens and preventing the development of T and B cell memory [348, 362]. Whilst there is evidence to suggest that the immune system cannot mount an effective response to unrelated antigens

during the first 21 days of an acute malaria infection, and that this is mediated by dendritic cell uptake of haemozoin [363], there is also evidence to suggest that the induction of immunity to the parasite itself is fully functional [265, 364]. Mice that have cleared primary infections are largely resistant to infection with homologous, and to varying degrees heterologous, parasites. Whilst it takes many infections for people to develop immunity to malaria this may well be due to the fine specificity of immunity to particular isolates. In short, immunity to isolates is acquired with a single infection but immunity to the whole species is built up over many infections. This may be one of the reasons why producing an effective vaccine against blood-stage malaria has so far been difficult. An alternative hypothesis is that the malaria parasite produces a large number of strongly immunogenic antigens that are not essential to its survival, creating a smokescreen to hide those molecules that are essential (review [365]).

Chronic malaria infection has a depressive effect on the immune responses to other antigens including experimental antigens such as sheep erythrocytes [40]. Malaria infection can also alter the antibody isotype to non-malaria antigens, to IgM instead of IgG [366]. There could therefore be a short optimal time period between transmission seasons when vaccination in endemic areas would be most effective. Additionally, if malaria infection inhibits the development of T and B cell memory, it may suppress the efficacy of any vaccines to blood-stage malaria [367].

Clearance of parasitaemia of infection in humans is complicated by continual re-infection, resulting in chronic infection. This persistence of infection may be due to the predominance of strain-specific immunity particularly in *P.falciparum* infections [360, 368], the recrudescence of infection from hypnozoites [19] or to the concurrent infections

protecting the host from infection with other, perhaps more virulent, strains [369]. In other parasitic infections, such as *L. donovani*, low-level chronic infection is essential for the establishment of concomitant immunity [107], however low-level infection of *Brugia pahangi* and *Toxoplasma gondii* does not seem to protect hosts against re-challenge [213, 370]. The effect of chronic malaria on the development of a new malaria infection is unknown.

In order to investigate persistent infections, secondary infections and memory responses, it is first necessary to determine when primary infection is naturally cleared. This is an important aspect of the infection, which has not been investigated in any rodent model. The reasons for persistence of some strains of parasites and not others are unknown, however they are likely to include variation of parasite antigens to avoid the hosts' immune system [365] and a trade-off between the fitness cost to the host and the virulence and replication rate of the parasite [31]. Ultimately, parasite persistence increases the likelihood of transmission.

Aims of the thesis

This introduction has shown that despite the importance of the spleen in immunity to malaria, and the demonstrated changes in splenic architecture, the mechanism of these changes is still unknown. The extent, order and timing of these changes has also not been fully characterised. Immunity to malaria does occur in the field and B cells are known to be important in that immunity. However, the development and maintenance of good B cell and antibody responses has still not been well characterised, and it has been debated whether or not they form. The mechanism of B cell memory maintenance, whether by production of long-lived cells or by continuous turnover of short-lived cells, is also

controversial, yet despite investigations into B cell responses in malaria the chronicity of infection in model systems has not been determined.

This thesis aims to pinpoint the order and timing of changes in splenic microarchitecture during acute *P. c. chabaudi* infection, follow the development and maintenance of memory B cells and plasma cells during and after chronic infection, and to determine the natural clearance point of chronic infection.

Objectives:

1) To determine the duration of a chronic *P. c. chabaudi* infection
2) To conduct a detailed analysis of splenic microarchitecture and cellular composition during the acute infection
3) To assess longevity and turnover of B cells and plasma cells in relation to antibody titres
4) To assess longevity and turnover of B cells and plasma cells during chronic infection

Chapter 2

Buffers

Alkaline phosphate substrate buffer

100mM Tris-HCl	(Sigma-Aldrich Inc; T3253)
100mM NaCl	(Fisher; 7647-14-5)
5mM $MgCl_2$	(Sigma-Aldrich Inc; M8266)
pH 9.5	

5-bromo-4-chloro-3-indolyl-phosphate (BCIP) stock

BCIP	100mg (Promega; S381C)

Add 2ml of 100% dimethylformamide (Sigma-Aldrich Inc; D4254)

Coomassie blue

0.25% w/v Coomassie Brilliant Blue	(Sigma-Aldrich Inc; B-0149)
45% ethanol	(Fisher; E/0560DF/17)
10% acetic acid	(BDH; 10002)
	Make up to final volume with deionised H_2O

Coomassie blue destain

45% ethanol	(Fisher; E/0560DF/17)

10% acetic acid	(BDH; 10002)
	Make up to final volume with deionised H_2O

DNA extraction buffer

50mM Tris-HCl	(Sigma-Aldrich Inc; T3253)
50mM EDTA	(Sigma-Aldrich Inc; E5134)
100mM NaCl	(Fisher; 7647-14-5)
pH7.5	

FACS buffer

500ml PBS	(T/C grade; Gibco/Life Technologies; 20012-019)
1% BSA	(Sigma-Aldrich Inc; A9647)
0.005M EDTA	(Sigma-Aldrich Inc; E5134)
Sterile filter before use	

Giemsa buffer

0.09% w/v NaCl	(Fisher; 7647-14-5)
0.02mM KH_2PO_4	(BDH; 102034B)
0.08mM K_2HPO_4	(BDH; 296194X)
pH 7.0 – 7.2	

Giemsa stain

Giemsa R66 solution	(BDH; 352603R)
Dilute 1 in 10 in Giemsa buffer	

Histology buffer

Tris pH 7.6 (0.05M Tris)	1.6 parts saline stock, 1 part Tris stock, 1.4 parts water with 1% HCl (BDH; 101254H)
Tris stock (0.2M)	121.14g Trizma base (Sigma-Aldrich Inc; T6066) in 5L deionised water
Saline stock	42.5g NaCl (Fisher; 7647-14-5) in 5L deionised water

Kreb's glucose saline

55mM NaCl	(Fisher; 7647-14-5)
4.6mM KCl	(Sigma-Aldrich Inc; P3911)
2.4mM $MgSO_4.7\ H_2O$	
Buffer pH7.4	222.2ml
26mM NaH_2PO_4	(BDH; 102454R)
NHCl	4.36ml
dH_2O	217.86ml
dH_2O	777.8ml
11mM Glucose	

MSP-1 purification – elution buffer

500ml PBS	(T/C grade; Gibco/Life Technologies; 20012-019)
50mM NaH_2PO_4	(BDH; 102454R)

300mM NaCl (Fisher; 7647-14-5)

250mM imidazole (Sigma-Aldrich Inc; 10250)

pH 8.0

MSP-1 purification – lysis buffer

500ml PBS (T/C grade; Gibco/Life Technologies; 20012-019)

50mM NaH_2PO_4 6.9g/l (BDH; 102454R)

300mM NaCl 17.54g/l (Fisher; 7647-14-5)

10mM imidazole 0.68g/l (Sigma-Aldrich Inc; 10250)

pH 8.0

MSP-1 purification – wash buffer

500ml PBS (T/C grade; Gibco/Life Technologies; 20012-019)

50mM NaH_2PO_4 6.9g/l (BDH; 102454R)

300mM NaCl 17.54g/l (Fisher; 7647-14-5)

20mM imidazole 1.36g/l (Sigma-Aldrich Inc; 10250)

pH 8.0

Nitro-Blue Tetrazolium (NBT) stock

NBT 100mg (Promega; S381C)

Add 2ml of 70% dimethylformamide (Sigma-Aldrich Inc; D4254)

Parasite freezing medium

40% glycerol	(Sigma-Aldrich Inc; G5516)
0.14M Sodium lactate	(Sigma-Aldrich Inc; L7022)
0.005M KCl	(Sigma-Aldrich Inc; P3911)
pH 7.4	

SDS-PAGE reducing loading buffer

1M Tris-HCl	8ml (Sigma-Aldrich Inc; T3253)
10% w/v SDS	20ml (BioRad; 161-0418)
20mM dithiothreitol (DTT)	154.2mg
Glycerol	10ml (Sigma-Aldrich Inc; G5516)
0.2% w/v bromophenol in ethanol	0.6ml (Sigma-Aldrich Inc; B5525)
	Make up to 50ml with deionised H_2O

SDS-PAGE running buffer

NuPAGE MES SDS running buffer (20x)	(Invitrogen; NP0002)
Dilute 1:20 in MilliQ H_2O	

Semi-supplemented HBSS

500ml Hank's balanced salt solution	(Gibco; 24020-091)

12mM Hepes (Gibco; 15630-056)

Supplemented HBSS

500ml Hank's balanced salt solution (Gibco; 24020-091)

5% FCS

12mM Hepes (Gibco; 15630-056)

Tris buffer pH 9.2

0.02M Tris (Sigma-Aldrich Inc; T6066)

0.12M NaCl (Fisher; 7647-14-5)

0.03M HCl (BDH; 101254H)

Western blot alkaline phosphatase substrate

5ml Alkaline Phosphatase substrate buffer

33µl NBT stock

17µl BCIP stock

Western blot transfer buffer

24mM Tris (Sigma-Aldrich Inc; T6066)

0.19M Glycine (BDH; 28458)

Methanol 200ml (Romil; H410)

Make up to 1L with dH_2O

Suppliers

Amersham Biosciences – GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA

Amicon – Millipore UK Ltd., Units 3 & 5 The Courtyards, Hatters Lane, Watford WD18 8YH

Animalcare Ltd., Common Road, Dunnington, York YO19 5RU

Applied Biosystems, 850 Lincoln Centre Drive, Foster City CA 94404 USA

Avid Identification Systems Inc., 3185 Hamner Ave, Norco CA 92860

Axxora (UK) Ltd., PO Box 6757, Bingham, Nottingham NG13 8LS

BD, 21 Between Towns Road, Cowley, Oxford OX4 3LY

BDH – VWR International bvba, Haasrode Research Park, Zone 3, Geldenaaksebaan 464, B-3001 Leuven

BioRad Laboratories Ltd., Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX

Bright Instruments Co. Ltd., St. Margarets Way, Huntingdon, Cambridgeshire PE29 6EU

Corning, One Riverfront Plaza, Corning, NY 14831 USA

Dako Cytomation, 6392 Via Real, Carpinteria, CA 93013 USA

Euro-Diagnostica BV, Beijerinckweg 18, NL-6827 BN Arnhem, The Netherlands

Falcon – BD, 21 Between Towns Road, Cowley, Oxford 0X4 3LY

Fisher Scientific Ltd., Bishop Meadow Road, Loughborough, Leicstershire LE11 5RG

Gibco – Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF

Harlan UK Ltd., Shaw's Farm, Blackthorn, Bicester, Oxon OX25 1TP

Hawksley, Marlborough Road, Lancing Business Park, Lancing, Sussex BN15 8TN

CA Hendley (Essex) Ltd., Oakwood Hill Industrial Estate, Loughton, Essex

Hybaid – Thermo Electron, 171 Industry Drive, Pittsburgh PA 15275

Invitrogen Ltd., 3, Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF

Kendall, 15 Hampshire Street, Mansfield MA 02048

Leo Labs, Princes Risborough, Buckinghamshire HP27 9RR

Miltenyi Biotec Ltd., Almac house, Surrey GU24 9DR

Molecular Probes – Invitrogen Ltd., 3, Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF

Nalge Nunc Int., 75 Panorama Creek Drive, Rochester NY 14625

PMI Nutrition Int., PO Box 19798, Brentwood, MO 63144 USA

Pierce – Perbio Science UK Ltd., Unit 9, Atley Way, North Nelson Industrial Estate, Cramlington, Northumberland NE23 1WA

Promega, Delta House, Southampton Science Park, Southampton SO16 7NS

Qiagen Ltd., Qiagen House, Fleming Way, Crawley, West Sussex RH10 9NQ

Raymond A Lamb Ltd., Units 4 & 5, Parkview Industrial Estate, Alder Close, Lottbridge Drove, Eastbourne, East Sussex BN23 6QE

Rhône-Mérieux Ltd., Spire Green Centre, Harlow, Essex CM19 5TS

Romil Ltd., The Source, Convent Drive, Waterbeach, Cambridge CB5 9QT

Rotring GmbH, PO Box 54, 10 60 D-22510, Hamburg

Sigma-Aldrich Inc., 3050 Spruce St., St. Louis, MO 63103 USA

Thermo Electron, 171 Industry Drive, Pittsburgh PA 15275 USA

Treestar Inc., 340 A Street Bd1 #203, Ashland, OR 97520

Vericore Ltd., Kinnoull Road, Dunsinane Industrial Estate, Dundee DD2 3XR

Materials and Methods

Mice

Female C57BL/6, BALB/c, Rag $1^{-/-}$ mice on a C57BL/10 background [371] and male Rag $2^{-/-}$ mice on a BALB/c background [372] bred in the SPF unit at the National Institute for Medical Research were used at 6-12 weeks of age. All mice were conventionally housed on sterile bedding, food and water. The food was autoclaved CRM rodent diet (Harlan) until 2004 and was then changed to Lab diet 5021-3 (PMI Nutrition Int.). For short-term experiments, animals were identified by tail marking with a waterproof Edding pen. For long-term experiments, 12-mm microchips (Avid) were implanted for identification. The light cycle consisted of 12 h light and 12 h dark to mimic conditions in malaria-endemic regions. For schizont enrichment, mice were kept on a reverse light cycle where daylight occurred from 8pm to 8am. For identification of cells dividing at specific time-points, mice were given 5-Bromo-2′-deoxyuridine (BrdU) 0.8mg/ml in drinking water, changed daily for 14 or 28 days. Mice were checked daily to identify gross health problems and cages were cleaned once a week.

Parasites

Plasmodium chabaudi chabaudi (AS) was originally isolated from an African thicket rat (*Thamnomys rutilans*) by Dr. D. Walliker and cloned *in vivo* by passaging in mice [373]. A stock of this was obtained from Dr. K. N. Brown, NIMR. Frozen stocks were negative for mycoplasma and for common mouse pathogens such as Hepatitis, Pasteurella and Lactate Dehydrogenase Virus. To make a new stock, one vial was thawed, diluted with 0.9% saline (Sigma-Aldrich Inc) and injected into several (e.g. between 6 and 10) two to three month

old C57BL/6 female mice. Such amplification of stock reduces mutation and genetic drift to a minimum, ensuring that each infection is as similar as possible to each other, and to the starting stabilate. One to two days before the peak of parasitaemia (approximately 10-15% parasitaemia), mice were anaesthetised by injection of 0.1-0.15ml Sagatal (pentobarbitone sodium BP; Rhône-Mérieux; 7SA005; 60mg/ml), 0.05ml Euthanal (pentobarbital sodium Ph.Eur; Vericore Ltd; 05327/4112; 200mg/ml) or Pentoject (pentobarbitone sodium Ph.Eur; Animalcare Ltd.; XUD132; 200mg/ml). Blood was taken after cardiac puncture with a syringe and transferred to a 15ml tube (Falcon) containing 50µl heparin per mouse (Leo Lab; 9860; 5000U/ml) on ice. Blood was centrifuged (10 mins, 1064 x g, 4°C), plasma removed and the pellet diluted with two pellet volumes sterile parasite freezing medium. Blood was aliquoted into cryotubes, frozen quickly at –80°C then transferred to liquid nitrogen for long-term storage. For initiation of infection, parasitised red blood cells (pRBC) were routinely injected intra-peritoneally (i.p.) from stabilate stocks kept in liquid nitrogen, thawed and diluted 1:1 with 0.9% saline. All injections were performed with sterile 1 ml syringes (BD) and 27G x $^1/_2$" needles (Kendall). Parasites were passaged up to four times in mice by i.p. injection of 10^6, 10^5 or 10^4 pRBC diluted in 100µl 0.9% saline (Sigma Aldrich Inc.) or Kreb's glucose saline [41]. Inoculum sizes were calculated by determining the percentage of parasitaemia in the infected mice and assuming an erythrocyte density of 6 x 10^9 / ml blood. pRBC were obtained from infected mice before the peak of parasitaemia, with parasitaemia determined by Rapi-Diff II stain (Raymond Lamb) of thin blood films made with blood from a small tail nick. For experimental infection, mice were injected with 10^5 pRBC diluted in 100µl 0.9% saline or Kreb's

glucose saline. For secondary infections, mice were injected with 10^5 pRBC diluted in 100µl 0.9% saline 3 months after the start of the primary infection.

Giemsa staining of thin blood films

Thin blood films were air dried and fixed with 100% methanol (BDH). Giemsa R66 solution (BDH) was diluted 1:10 in Giemsa buffer. Slides were immersed in staining solution for 20 minutes, rinsed with water and air dried. Slides were analysed under oil immersion on a Zeiss Axioskop light microscope with a 100x objective (final magnification 1200x). For high parasitaemia (over 1%) at least 10 fields, defined by a grid in the eyepiece, were counted. For low parasitaemias (less than 1%) at least 50 fields were counted.

$$\% \text{ parasitaemia} = \frac{\text{Total number pRBC}}{\text{Number RBC in a representative field x Number of fields counted}} \times 100$$

Schizont purification and CFSE labelling of schizont-infected red blood cells

Mice just before the peak of parasitaemia were anaesthetised with 100µl Sagatal. Blood was taken from the peritoneal cavity after cardiac puncture with a syringe containing 50µl heparin and kept on ice. A MACS column (Miltenyi; Type D; 130-041-201) and stop-cock were assembled on a clamp stand. A 50ml syringe was used to wash the column with dH_2O 2-3 times, then with RPMI (Gibco) 2-3 times. The column was placed in the column holder on a SuperMACS II magnetic separator. Mature malaria parasites containing the iron-rich molecule haemazoin are attracted by the magnet and remain on the column whilst uninfected red blood cells pass through the column [314, 374]. Blood samples (~20ml)

were made up to at least 30ml with RPMI, then loaded onto the top of the column. The column flow rate was adjusted to a steady drip (approx. 1 drip in 2 seconds) and flow-through was collected into 50ml tubes (Falcon). 1-2 x 50mls RPMI was also loaded onto the column to rinse it. The flow was stopped and the column removed from the magnet. The lid was placed on the column and a 50ml syringe used to flush the schizont-enriched blood into a 50ml tube. The column was then washed with RPMI, dH_2O and 100% ethanol before storing in 100% ethanol (BDH; 10107). The schizonts were centrifuged at 804 x g for 5 mins at 4°C, the supernatant was removed, the pellet resuspended and a thin blood film made to check the purification. Erythrocytes were counted using a haemocytometer, then schizonts were diluted to approximately 6.667 x 10^6/ml in PBS. 1µl CFSE (Molecular Probes) per ml of schizont solution was added from a 5mM stock in dimethyl sulphoxide (DMSO, Sigma-Aldrich Inc) and the cells incubated for 5 mins at room temperature. Cells were washed 3 x in RPMI then resuspended in RPMI and injected i.v. into C57BL/6 mice.

Subinoculation of blood

1-6 months after primary infection mice were anaesthetised by injection of 0.1-0.15ml Sagatal. Mice were exsanguinated by cardiac puncture with a syringe containing 200U/ml heparin in 250µl Kreb's glucose saline. Blood from one donor animal was injected i.p. into two immunodeficient (C57BL/10 Rag $1^{-/-}$ or BALB/c Rag $2^{-/-}$) mice, which were then monitored for parasitaemia by microscope analysis of Giemsa stained thin blood films as above, and killed by cervical dislocation once parasitaemia was ascertained. Mice not developing parasitaemia after 3 weeks were assumed not to have received viable parasites, and were culled.

Limiting dilution of parasites

pRBC were taken from a mouse before the peak of infection and serially diluted in 0.9% saline to a final concentration of 1, 10, 100 or 1,000 pRBC per 100μl, then injected i.p. into Rag $2^{-/-}$ mice, which were monitored for parasitaemia as above.

Administration of Indian Ink

Mice were injected intravenously (i.v.) with 200μl 5% Indian Ink (Rotring) in 0.9% saline.

Immunisation and blood sampling

Purified protein (MSP-1, CGG and pMCK110) at a concentration of 1mg/ml was diluted 1:1 with 9% potassium aluminium sulphate (Alum) and precipitated with 1M sodium hydroxide solution added dropwise until maximum precipitation was reached, as judged by eye. The precipitate was centrifuged for 5 mins at 289 x g, washed twice with PBS and resuspended in 0.9% saline to a final concentration of 250μg/ml. Mice were injected i.p. with 100μl of this solution. Mice immunised with MSP-1 and pMCK110 were boosted twice at two week intervals.

Treatment of mice with agonistic anti-lymphotoxin β antibody

Mice were infected with 10^5 pRBC in 100μl Kreb's glucose saline. On day 4 of infection, one third were injected with 50μg anti-lymphotoxin β receptor agonist antibody (Axxora) and one third with 50μg control rat IgG (Sigma-Aldrich Inc) in 0.9% saline. Parasitaemia was monitored by Giemsa-stained thin blood films as above. Half the mice from each group were killed by cervical dislocation for spleen harvesting (see below) on day 10 of primary infection. Remaining mice were given a secondary infection of 10^5 pRBC in 100μl Kreb's glucose saline 3 months after primary infection, parasitaemia was monitored by

Giemsa-stained thin blood films and mice were killed for spleen harvesting on day 10 of secondary infection.

Organ harvesting and freezing

Mice were killed by cervical dislocation. Spleens were extracted, all fat was carefully removed and the tips cut off to give flat ends to make sectioning easier. Spleens were placed on strips of aluminium foil, which was repeatedly dipped in liquid nitrogen to freeze the organs. Frozen organs were stored at –70°C.

Cryosectioning

A circle of Whatman filter paper was wetted and frozen onto the cryostat chuck. OCT compound (BDH) was placed on the filter paper and spleens mounted in, but not covered by, the OCT. 5μm thick sections were cut with a cryostat (Bright) and mounted on 4-spot slides (Hendley). Slides were air dried for 1 hour, fixed in cold (4°C) 90% acetone (BDH) for 20 mins and stored at –20°C.

Immunofluorescence histology

Slides were defrosted and rehydrated for 5 mins in histology buffer. Primary antibodies were diluted in histology buffer and centrifuged (10,000 x g, 10 mins) to remove aggregates. Slides were wiped dry around sections after each washing step and 75μl of diluted antibody added for each incubation step. Slides were incubated for 1 hour in the dark at room temperature, during which time secondary antibodies were pre-incubated with equal volume of normal mouse serum, diluted in histology buffer for at least 30 mins to prevent secondary antibodies cross-reacting with mouse immunoglobulin. All incubation steps were carried out in a box containing damp tissue to maintain humidity and prevent sections from drying out. Slides were washed 2 x 5 mins in histology buffer then incubated

with secondary antibody for 1 hour in the dark at room temperature. During this time Neutravidin Texas Red (if used) was pre-incubated with equal volume of normal mouse serum, diluted in histology buffer for 30 mins. Slides were washed again for 2 x 5 mins, then incubated with Neutravidin Texas Red for 2 hours in the dark at room temperature. Slides were washed for 2 x 5 mins in histology buffer and 1 x 5 mins in dH_2O, then mounted with fluorescence mounting medium (Dako). Antibodies used are listed in Table 3. Normal mouse serum was obtained by exsanguination of naïve C57BL/6 mice and serum extraction.

Immunohistology

For horseradish peroxidase (HRP) staining, slides were blocked with 2% H_2O_2 for 1 hour. Slides were incubated with primary antibodies as described above, washed and incubated with secondary antibodies for 45 mins. During this time Streptavidin ABComplex alkaline phosphatase (AP) (Dako) components were pre-incubated with an equal amount of normal mouse serum, diluted in histology buffer. Slides were washed, then incubated with Streptavidin ABComplex for 30 mins. For HRP staining, one tablet of 3, 3-diaminobenzadine tetrachloride (DAB) (Sigma-Aldrich Inc) was dissolved in 15ml Tris buffer pH 9.2, then filtered through Whatman filter paper before use, 10ml were collected and 0.1% H_2O_2 added. Slides were washed (2 x 5 mins in histology buffer, and two drops of DAB solution added to each section. Sections were incubated until staining became visible under a light microscope, then slides were washed for 2 x 5 mins in histology buffer. For AP staining, 8mg levamisole (Sigma-Aldrich Inc) was dissolved in 10ml Tris buffer pH 9.2. 3.5-4mg napthol AS-MX phosphate (Sigma-Aldrich Inc) was dissolved in 380µl N,N-dimethylformamide (DMF, Sigma-Aldrich Inc), then added to the levamisole

solution, followed by 10mg Fast Blue (Sigma-Aldrich Inc). The solution was filtered through Whatman filter paper before use, and two drops added to each section. Sections were incubated until staining became visible under a light microscope, then slides were washed for 2 x 5 mins in histology buffer and 1 x 5 mins in dH_2O. Slides were mounted with Immunomount (Thermo Electron).

Colour analysis of immunohistology images

Immunofluorescence images were acquired on a Leica confocal microscope and prepared for presentation using Adobe Photoshop. Colour spectrum analysis was done in ImageJ using the colour picker plugin (Daan Zhu, NIMR). Parameters for the colour spectrums were confirmed with the colour masker produced by the plugin during analysis.

MSP-1 protein production and purification

Recombinant MSP-1_{19} was expressed in *Pichia pastoris* SMD1168 strain with a His-tag by Dr. M. Hensmann as described [375]. 25µl glycerol stock of this construct was cultured overnight at 30°C in 5ml Yeast Extract Peptone Dextrose (YEPD) medium, plated out on YEPD agarose plates with Geneticin antibiotic (Gibco) at concentrations from 0.5-4 mg/ml and incubated for 4 days at 30°C. Once colonies had formed, a 10 or 20 litre culture was grown up in 1% glycerol stock for 24 hours, then protein expression was induced by switching to 0.5% methanol for 48 hours. The culture supernatant was harvested, concentrated to 450ml, aliquoted into 50ml tubes (Falcon) and stored at -20°C.

For MSP-1 purification, the supernatant was defrosted, adjusted to pH 7.0 and filtered through Whatman filter paper. Glass columns were loaded with Ni-NTA agarose (Qiagen), approximately 3ml agarose per 50ml supernatant. The column was washed with 3-5x column volume of wash buffer, then the supernatant was passed through the column and

the flow-through collected. The column was washed with 3-5x column volume of wash buffer and 3-5x column volume of lysis buffer until the OD_{280} of the flow-through was the same as the OD_{280} of the lysis buffer. The protein was eluted with 2x column volume of elution buffer, then the eluate was concentrated with a Centricon YM3 (Amicon) at 3,000 x g and dialysed against PBS. Final protein concentration was measured spectrophotometrically at OD_{280}.

Recombinant MSP-1 protein pMCK110 (Figure 9) was expressed in *Escherichia coli* (*E.coli*) as a fusion protein with maltose binding protein (MBP), extracted and purified as described [376].

Protein gels and Western blots

Denaturing SDS-PAGE was performed under reducing conditions according to the Laemmli method as described in Current Protocols in Immunology (John Wiley and Sons Inc., 1995). Pre-made 10% Bis-Tris polyacrylamide gels (Nunc) were removed from their packaging, rinsed with dH_2O and placed in an electrophoresis tank with running buffer. 10µl protein was diluted 1:1 with SDS-PAGE reducing loading buffer and heated for 5 mins at 95°C to denature the proteins. Samples were loaded into wells in the gel and a prestained protein molecular weight standard with a range of approximately 10-250kDa (Biorad) was included. A constant voltage of 120 V was applied to the gel and progress of the proteins was monitored by the location of the bromophenol blue band. Proteins were visualised by staining with Coomassie blue. The gel was covered with Coomassie staining solution and placed on shaker for 1 h at room temperature or until the gel was fully stained. After this, the gel was rinsed in deionised water followed by incubation with destain solution. The gel was placed on a shaker until the protein bands became visible in blue and

the background was clear or only moderately stained. The destaining solution was then replaced by deionised water. Gels were photographed, then dried onto Whatman filter paper with a Scie-Plas gel drier (model GD4534) for long-term storage.

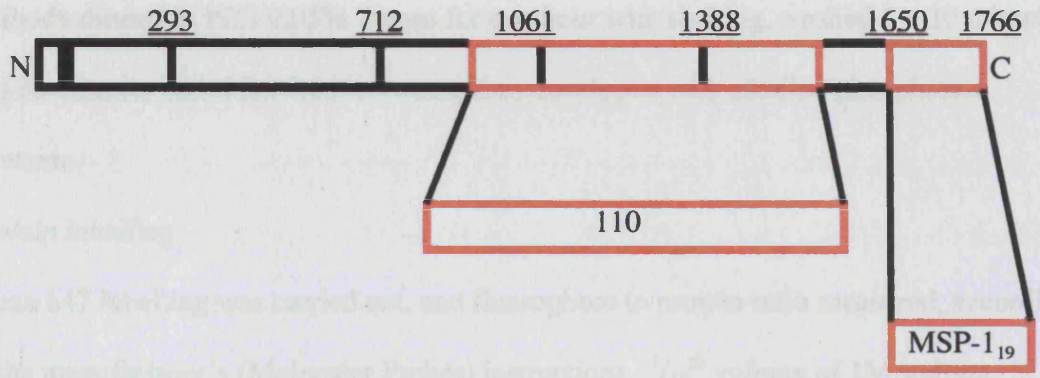


Figure 9. Full length *P.chabaudi* MSP-1 molecule and recombinant proteins. Full length protein (□), and two recombinant proteins [375, 376], that were labelled with fluorescent probes for detection of antigen-specific B cells and plasma cells (□). Numbers underlined indicate location of natural proteolytic cleavage sites. Figure adapted from [377]

For Western blotting, gels were placed on a piece of nitrocellulose protein transfer membrane (Hybond-C extra; Amersham Biosciences). Gels and paper were sandwiched between pieces of Whatman filter paper (4 pieces each side) and sponge pads. These were placed in a clamp in a transfer tank with transfer buffer, with the nitrocellulose membrane closest to the positive terminal. All pieces of filter paper, sponge and nitrocellulose paper were pre-soaked in transfer buffer before touching gels. A

constant voltage of 40V was applied for 1 hour. The clamp was removed and the gel and filter paper discarded. The nitrocellulose membrane was blocked in PBS (5% BSA) overnight. The membrane was washed for 6 x 5 mins with PBS 0.05% Tween and incubated with antibody diluted in PBS 0.05% Tween 1% BSA for 1 hour, then washed 6 x 5 minutes with PBS 0.05% Tween. The membrane was then incubated with a secondary antibody diluted in PBS 0.05% Tween for one hour with shaking, washed 6 x 10 minutes and 30 minutes with PBS 0.05% Tween, then developed with alkaline phosphatase substrate.

Protein labelling

Alexa 647 labelling was carried out, and fluorophore to protein ratio measured, according to the manufacturer's (Molecular Probes) instructions. $^{1}/_{10}{}^{th}$ volume of 1M sodium bicarbonate was added to the (1-2mg/ml) protein solution, which was then transferred to the vial of reactive dye. The protein and dye were incubated for 1 hour at room temperature then excess dye was removed by passing the solution over a spin column, which retains free dye on the column. The absorbance of the solution was measured at 280nm and 650nm by a spectrophotometer, and the fluorophore to protein ratio was calculated as follows:

$$\text{Protein concentration (M)} = \frac{(OD_{280} -)OD_{650} \times 0.03)) \times \text{dilution factor}}{203{,}000}$$

$$\text{Moles dye per mole protein} = \frac{OD_{650} \times \text{dilution factor}}{239{,}000 \times \text{protein concentration (M)}}$$

Flow cytometry

Mice were killed by cervical dislocation, spleens and bones extracted and placed in supplemented Hank's Buffered Salt Solution (HBSS). For collagenase treatment, spleens were placed in a Petri dish (Corning) with 3ml semi-supplemented HBSS, 1.65ml collagenase and 0.5ml DNase, cut into small pieces and incubated at 37°C, 7% CO_2 for 30 mins. For preparation without collagenase, spleens were mashed through a 0.7μm sieve (Falcon) into supplemented HBSS using the plunger of a sterile 5ml syringe (BD). Spleen cell suspensions were centrifuged at 289 x g for 10 mins at 4°C, washed with HBSS and centrifuged again. Bone marrow was flushed out with supplemented HBSS and centrifuged at 289 x g for 10 mins at 4°C. The supernatant was removed, the pellet resuspended and red blood cell lysis buffer (Sigma-Aldrich Inc.) was added, 2ml for suspensions from small spleens or bone marrow and 5ml for suspensions from large infected spleens, and cells were incubated at room temperature for 10mins. Supplemented HBSS was added to 20ml to stop the reaction, cells were centrifuged (289 x g, 10mins, 4°C) and resuspended in FACS buffer. Live cells were counted using a haemocytometer (Hawksley) and trypan blue (Sigma-Aldrich Inc.) exclusion, or on a Coulter Counter (10μl cell suspension in 10ml isotonic buffer). 1 x 10^6 cells per well were added to 96-well V-bottom microtitre plates (Nunc) and incubated for 10 mins with 25μl/well Fc receptor blocking antibody (24G2; [378]). Antibodies were diluted in FACS buffer, 25μl added to each well and incubated for 30 mins on ice in the dark. Plates were centrifuged for 5 mins at 289 x g, 4°C and washed twice with 150μl/well FACS buffer. Cells were fixed with 2% paraformaldehyde (Sigma-Aldrich Inc.) for 30 mins to 1 hour on ice in the dark, washed and resuspended in FACS buffer. For intracellular staining, after fixation cells were washed, permeabilised with 0.1%

NP-40 (nonylphenyl-polyethyleneglycol acetate; Sigma Aldrich Inc.) for 3 mins, centrifuged and incubated with 20μl/well anti-BrdU antibody with DNase (BD) or control antibody for 1 hour at room temperature, washed and resuspended in FACS buffer. Antibodies used are listed in Table 4. Cells were collected on a FACSCalibur (BD) and analysed with FlowJo (Treestar), Microsoft Excel and Graphpad Prism 4. Statistical significance was tested with a non-parametric, two-tailed Mann Whitney test.

Table 3. Reagents used for immunohistology

Reagent	Source	Catalogue no.	Clone	Isotype	Concentration/ dilution for use
Rat anti-mouse CD3 IgG	Serotec	MCA5006	KT3	IgG2a	1.25μg/ml
Rat anti-mouse IgM heavy chain	Serotec	MCA199			0.42μg/ml
Rat anti-mouse CD8a	BD	553029	53-6.7	IgG2a	5μg/ml
Hamster anti-mouse CD11c Alexa 488	Caltag	MCD11c20	N418	IgG	1μg/ml
Biotinylated hamster anti-mouse CD11c	BD	553800	HL3	IgG1	5μg/ml
Sheep anti-mouse IgD	Binding Site	PC283		IgG	0.135mg/ml
Anti-mouse CD138 (syndecan-1)	BD	09342D	281-2	IgG2a	0.255μg/ml
Biotinylated peanut agglutinin	Vector Labs	B-1075			50μg/ml
Rat anti-mouse ER-TR9	BMA	T-2010	ER-TR9	IgM	2.5ug/ml
Rat anti-mouse MAdCAM-1	Serotec	MCA1551	MECA-367	IgG2a	2.5ug/ml
Rat anti-mouse MOMA-1	Serotec	MCA947	MOMA-1	IgG2a	1/10
Rat anti-mouse CD169	Serotec	MCA884	3d6.112	IgG2a	2.5μg/ml
Rat anti-mouse F4/80 FITC	eBioscience	11-4801	BM8	IgG2a	1.25μg/ml
Donkey anti-sheep IgG HRP	Binding Site	AP360			1/100
Biotinylated rabbit anti-rat IgG	Dako	E0468			1/600

Reagent	Source	Catalogue no.	Clone	Isotype	Concentration/ dilution for use
Donkey anti-sheep IgG Alexa 647	Molecular Probes	A-21448		IgG	10μg/ml
Donkey anti-sheep IgG Alexa 568	Molecular Probes	A-21099		IgG	
Goat anti-rat IgG Alexa 488	Molecular Probes	A-11006		IgG	6.67μg/ml
Goat anti-rat IgM Alexa 488	Molecular Probes	A-21212		IgG	6.67μg/ml
Chicken anti-rat IgG Alexa 647	Molecular Probes	A-21472		IgG	6.67μg/ml
Rat anti-mouse Fc receptor	NIMR		24G2	IgG1	1:5
Rat anti-mouse pDC Alexa 488	G.Trinchieri		120G8	IgG1	2μg/ml
Goat anti-mouse IgM (μ chain) FITC	Sigma	F-9259			1:400
Goat anti-mouse IgG (γ chain) FITC	Sigma	F-8264			1:400
Rat anti-mouse ER-TR7	BMA	T-2109	ER-TR7	IgG2a	1.33μg/ml
Rat anti-mouse CD31 FITC	BD	553372	MEC 13.3	IgG2a	0.83μg/ml
Hamster anti-mouse LTβ	BD	552938	BB.F6.F6.BF2	Arm hamster	1μg/ml
Hamster anti-mouse LTβR	BD	552939	AC.H6	Arm hamster	1μg/ml
Mouse anti-hamster IgG biotinylated	BD	554010	G70-204 & G94-56	IgG1 & IgG2b	1:100
Tyramide signal amplification kit	Molecular Probes	T20932			

Table 4. Antibodies and labelled antigens used for flow cytometry

Reagent	Source	Catalogue no.	Clone	Isotype	Concentration/ dilution for use
Rat anti-mouse CD19 biotinylated	BD	553784	1D3	IgG2a	0.5μg/ml
Rat anti-mouse CD138 PE	BD	553714	281-2	IgG2a	1μg/ml
Rat IgG2a isotype control biotinylated	BD	553928	R35-95	IgG2a	0.5μg/ml
Rat IgG2a isotype control PE	BD	553930	R35-95	IgG2a	1μg/ml
Mouse anti-BrdU FITC with DNase	BD	350649		IgG1	20μl per well
Mouse IgG1 isotype control FITC	BD	550616	MOPC-31C	IgG1	5μg/ml
pMCK110 Alexa 647	NIMR				200μg/well
CGG Alexa 647	NIMR				200μg/well
pMCK110 FITC	NIMR				200μg/well
CGG FITC	NIMR				200μg/well
Rat anti-mouse Fc receptor	NIMR		24G2	IgG1	1:5
Rat anti-mouse IgM (μ chain) APC	BD	550676	11/41	IgG2a	1:400
Goat anti-mouse IgG (γ chain) FITC	Sigma	F-8264			1:400
Streptavidin Tricolour	BD	SA1006			1:100
Rat anti-mouse IgD FITC	BD	553439	11-26c.2a	IgG2a	10μg/ml
Goat anti-hamster IgG FITC	Jackson	127-095-160			1:400
Rat anti-mouse CD19 APC	BD	550992	1D3	IgG2a	2μg/ml
Rat anti-mouse CD19 PE	BD	553786	1D3	IgG2a	2μg/ml

Chapter 3

Determining the duration of a primary infection of Plasmodium chabaudi chabaudi (AS)

Introduction

Malaria is a chronic infection in its natural host. A single infection with either *Plasmodium falciparum* or *Plasmodium vivax* causes a long-term recrudescing infection that can persist for several years in the absence of re-infection [379], although in endemic areas this pattern of infection is often complicated by many re-infections over the course of the transmission season [380]. Such multiplicity of infection is associated with a reduced risk of clinical disease [369]. Once the infection is cleared, however, immunity to re-infection wanes, so much so that absence from endemic areas for a long period of time results in loss of immunity [18]. The reasons for the existence of this concomitant immunity, and the lack of sterile immunity to malaria, are unknown. It is thought that the expression of variant antigens is a major contributor to this effect [360], however other mechanisms of immune evasion such activation of regulatory T cells [112] are also thought to have roles in the parasite's escape from immune responses (review [365]).

It is sometimes difficult to distinguish direct effects of the parasite from inadequate or inappropriate host responses. Nevertheless, it is clear that alterations in the function of the immune system do occur in malaria infection. For example, the high degree of alteration in

the splenic microarchitecture [345, 346] is likely to affect the development of immune responses to both malarial and other antigens. Alteration of immune responses can be seen in both the acute and chronic phases of malaria infection [40, 366, 381-384]. Defective antigen-processing caused by parasite-produced haemazoin has been suggested to play a role in immunosuppression [385], and alterations of dendritic cell function have also been suggested to contribute to malarial immunosuppression, although this is somewhat controversial (review [91]). These immunosuppressive effects of malaria have major implications for the development and severity of human infections, as well as for the efficacy of vaccination, as the majority of people in endemic areas will be infected with at least one other pathogen, more likely several different pathogens.

Plasmodium chabaudi, as well as *P.falciparum* and *P.vivax*, can produce a long-term recrudescing infection in its natural host [17]. Infection in laboratory mice does not exactly mimic the course of human infection, although it does have defined acute and chronic phases of disease, and the duration of laboratory infection has not been established. Malaria infection is often thought of as cured once parasitaemia can no longer be detected by Giemsa-stained thin blood film. It is possible, however, that the infection persists below this detection limit for many weeks.

In order to study responses to secondary infection, and also the effects of chronic malaria on the immune system, it is necessary to determine the exact time limit when live parasites are no longer present in the host. This chapter establishes the duration of primary infection in two common strains of laboratory mice, C57BL/6 and BALB/c, by sub-inoculation of blood from chronically infected mice to immunodeficient hosts.

Results

A typical course of parasitaemia in an infection of 1 x 10^5 *P.chabaudi* pRBC in C57BL/6 mice as described previously [265, 276, 293, 318, 346] is shown in Figure 10. The infection becomes patent, or detectable, usually at day 3, increases to a peak at day 8 then drops to less than 1% at around day 15. There is a slight recrudescence around day 20, then by day 30 the infection becomes subpatent – i.e. cannot be detected by thin blood film (detection limit approximately 0.001% or 5 parasites per μl blood).

In order to determine how long parasitaemia remains subpatent, infected mice were exsanguinated at different times after primary infection was initiated. The recipient mice chosen were immunodeficient Rag $2^{-/-}$ or Rag $1^{-/-}$ mice, which have no acquired immune system, as small numbers of parasites are more likely to grow in an immunodeficient than in a wild-type host. This was likely to detect low parasitaemias, since a single parasite can theoretically give rise to a full infection. The experimental plan is shown in Figure 11. Theoretically, a single parasite can give rise to a full infection, however parasite viability within the inoculum, even in immunodeficient hosts, is unknown. The route of infection is likely to affect efficacy; experimental infections are commonly given intra-peritoneally, which is likely to decrease parasite viability more than intra-venous infection. Therefore, the sensitivity of this method was tested by injecting known numbers of parasites (1-1,000) into immunodeficient mice, which were then monitored until the recipient mice became parasitaemic by Giemsa stained thin blood films. If parasitaemia was not detected within three weeks it was assumed that no parasitaemia would develop and the mice were culled. Time to detection of parasitaemia varied, however no parasitaemia was ever detected more

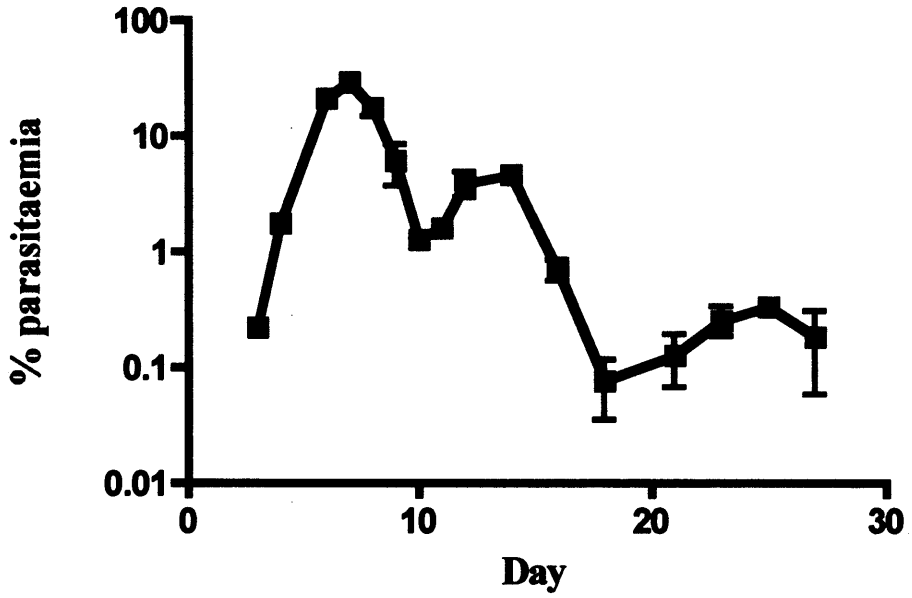


Figure 10. Representative course of primary parasitaemia in three C57BL/6 mice infected with 1 x 10^5 parasitised red blood cells. Error bars indicate standard error of the mean (SEM). Where error bars are not present, SEM is less than 10% of the mean.

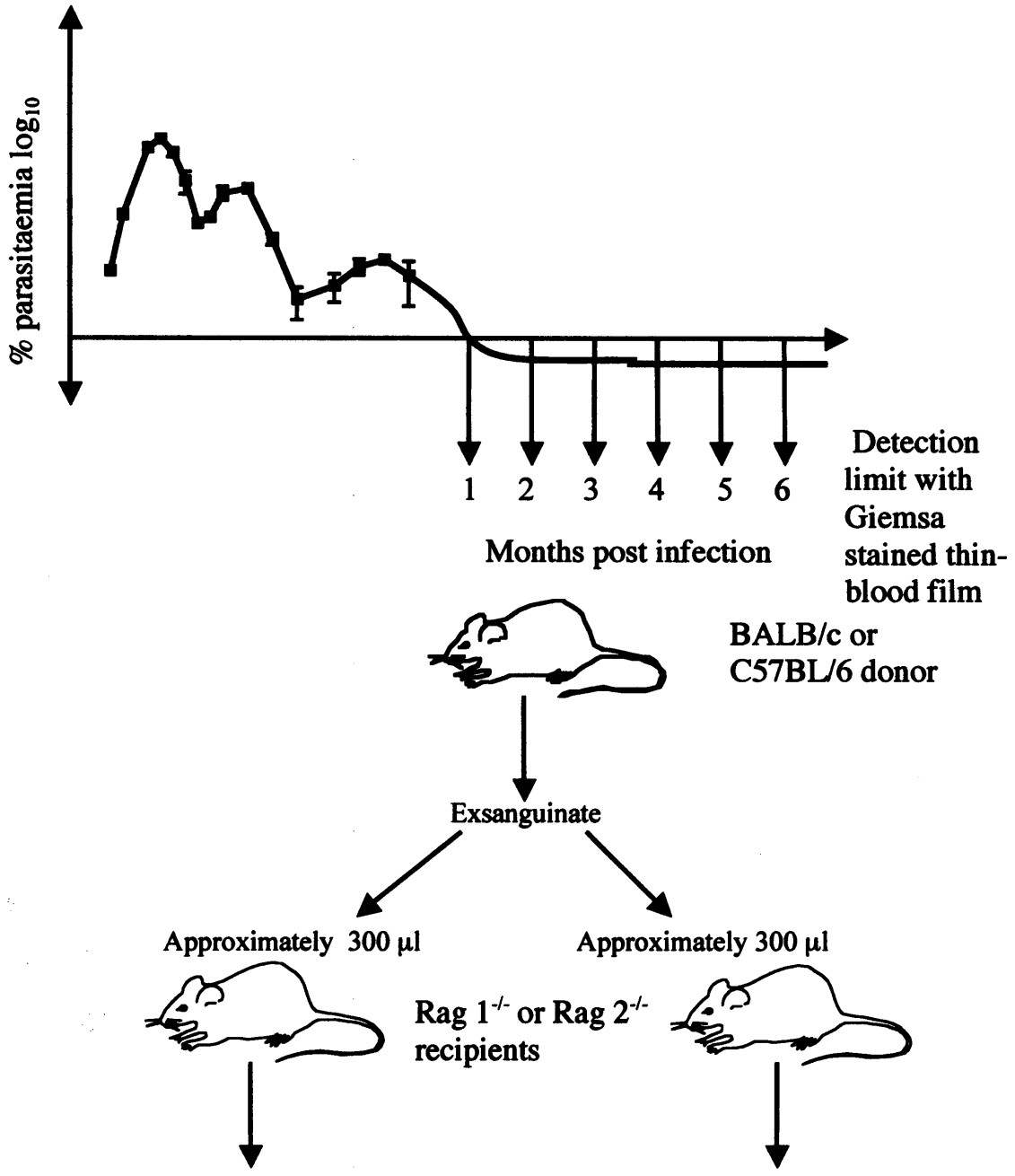


Figure 11. Experimental plan for sub-inoculation of blood from chronically infected mice to immunodeficient mice.

than 14 days after infection or sub-inoculation. The results of this limiting dilution experiment indicate that an infection of only 1 parasite can be reliably detected in 25% of recipients (Table 5). Parasites were not micro-manipulated, but were serially diluted in saline, and according to the zero order term of the Poisson distribution, with an average of 1 parasite per inoculum 66% of mice should develop parasitaemia, assuming that all parasites are viable and can grow in the new host. Therefore, out of 20 mice injected, 12 should develop parasitaemia if there is 100% efficacy. As only 5 of these mice became parasitaemic efficacy is <50%, nevertheless an inoculum of only 1 parasite can be reproducibly detected. In the sub-inoculation experiment, mice were given between 200 and 300μl of blood. The total blood volume of a laboratory mouse is approximately 2ml, therefore if we can detect a single parasite in $^{1}/_{10}^{th}$ this volume, we can detect a minimum of 10 parasites in the entire blood volume of a mouse. The sensitivity of this assay is therefore very high.

The results of the sub-inoculation of blood from chronically infected donors to immunodeficient recipients are shown in Table 6. From these results it appears that BALB/c mice clear their infection faster than C57BL/6 mice. The first time this result was obtained it was unexpected, therefore a second experiment was performed to test the validity of the results. The second experiment gave the same results as the first, demonstrating both the validity and reproducibility of these results.

Table 5. Limiting dilution of parasites

Number of parasites injected	% mice developing parasitaemia
1*	25% (5/20 mice)
10	80% (16/20 mice)
100	100% (10/10 mice)
1,000	100% (13/13 mice)

* Parasites from BALB/c were serially diluted in 0.9% saline to give an average of 1-1,000 parasites per 100µl injected volume, and injected into Rag $2^{-/-}$ mice

Table 6. Detection of residual parasitaemia by sub-inoculation of blood from chronically infected hosts to immunodeficient recipients

Time of blood transfer after primary infection	% of recipients developing detectable parasitaemia					
	Experiment 1		Experiment 2		Combined results	
	BALB/c	C57BL/6	BALB/c	C57BL/6	BALB/c	C57BL/6
1 month	100% (6/6)*	75% (6/8)	90% (9/10)	100 % (6/6)	100%	86%
2 months	0% (0/6)	33% (2/6)	0% (0/10)	27% (3/11)	0%	29%
3 months	0% (0/6)	0% (0/6)	0% (0/10)	0% (0/8)	0%	0%

* (x/y) indicates the number of mice developing parasitaemia (x) out of the total number of recipient mice (y)

Discussion

This study has established that the chronic phase of *P.chabaudi* infection can persist for up to 3 months, depending on the host mouse strain; and that sub-inoculation of blood from chronically infected mice to immunodeficient hosts is a sensitive method for determining subpatent infection, able to detect a single parasite in 300μl of blood. It has also shown that BALB/c mice clear residual parasitaemia faster than C57BL/6 mice.

Different immune responses to infection are well documented in BALB/c and C57BL/6 mice in response to *Leishmania* infection, where the Th2 response of BALB/c mice corresponds to susceptibility to disease, whilst the Th1 response of C57BL/6 mice corresponds to resistance to disease (review [386, 387]). These two mouse strains also differ in susceptibility to other infections (e.g. *Burkholderia*, *Trypanosoma*, ocular toxoplasmosis and viral infection) (review [388]) [262, 389-391]. This has been associated with higher levels of TNFα, nitrate and sometimes IFN-γ in C57BL/6 mice [389, 391], and decreased IL-12 and IL-12Rβ2 with increased IL-4 production in BALB/c mice [388], although higher levels of IFN-γ mRNA were found in BALB/c than in C57BL/6 mice in *Burkholderia pseudomallei* infection [390].

The difference in clearance of *P.chabaudi* parasites between BALB/c mice and C57BL/6 mice might be a consequence of a genetic predisposition of BALB/c mice towards a faster switch to Th2 responses, which might also mean faster and better antibody responses compared to C57BL/6 mice. Earlier production of antibody could be expected to correlate with faster clearance of parasites, since antibodies have been shown to be crucial for parasite clearance [287, 317, 318]. Higher IL-4 secretion from splenocytes of BALB/c

mice in response to *P.chabaudi* antigens has been observed [392], however a faster switch to a Th2 response in BALB/c mice may lead to increased susceptibility to higher acute parasitaemia, due to premature downregulation of the pro-inflammatory response before Th2 effector functions can be fully activated. In *P.falciparum* endemic areas, a polymorphism in the IL-4 promoter is associated with elevated anti-malaria IgG antibodies and decreased susceptibility to clinical infection [393]. In mice, the IgG2a isotype is associated with increased protection against *P.chabaudi* infection [329], however IL-4 non-responsive mice produce marginally lower levels of IgG1 switch transcripts and higher levels of IgG2a transcripts [394]. The effect of the timing and magnitude of the Th2 response on immune responses to malaria infection is, therefore, still unclear.

Chronic infection can allow the development of concomitant immunity (i.e. a low-level infection preventing the establishment of an acute infection from new exposure to the same pathogen), for example, maintenance of low-level infection of *Leishmania* is required for the persistence of immunity to re-infection [107]. Chronic infection can also affect the development of memory responses to the same pathogen, and can influence the amplitude and type of responses to other pathogens. Clearance of subpatent parasitaemia within 3 months in C57BL/6 mice is substantiated by other work done in our laboratory during the time of this thesis. C57BL/6 mice re-infected 2.5 months after primary infection (i.e. in the presence of chronic infection) had a lower peak parasitaemia than mice re-infected 4.5 months after primary infection (i.e. after clearance of chronic infection). Those mice re-challenged at 4.5 months after primary infection had higher parasitaemias, and also clear their parasites faster, whereas mice with lower secondary infection parasitaemias take longer to clear the infection to subpatent levels [395, 396] (Figure 12). This suggests the

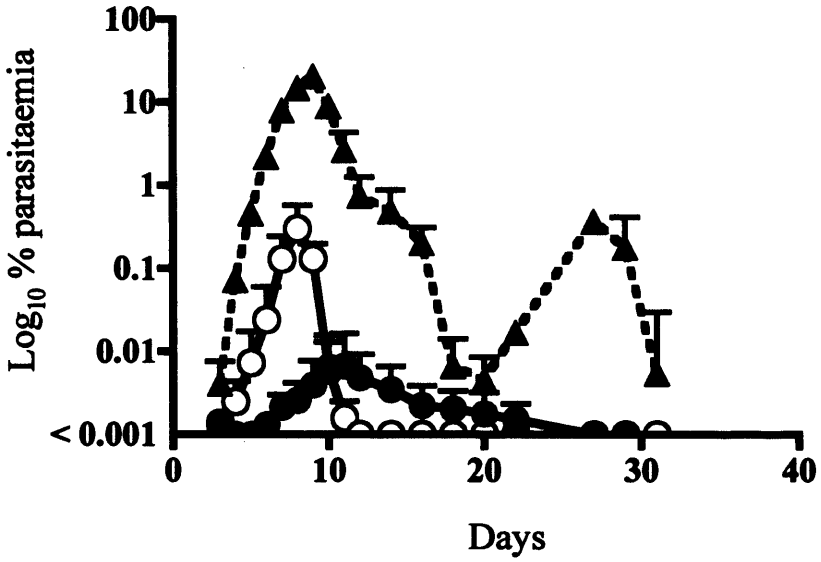


Figure 12. Variations in parasitaemia during primary infection (▲), and in secondary infection in the presence (●) or absence (○) of chronic parasitaemia.

A. Achtman, R. Stephens, E. Cadman, V. Harrison & J. Langhorne, manuscript submitted

existence of concomitant immunity in the C57BL/6 model of malaria. Malaria infections outside the laboratory often persist for the life of the host [17, 361, 397], although it is not clear to what degree this is due to continuous re-infection rather than persistence of a single infection. The precise effects of chronic infection on the development of memory responses are unclear, and the presence of memory T cells, memory B cells and long-lived plasma cells in malaria infection has not been demonstrated, but would be important to investigate.

Immunological memory is defined as the ability of the immune system to mount a more rapid and heightened response to antigens which have been encountered previously [43], and is thought to be mediated by a pool of antigen-specific cells that are more numerous than those present during primary exposure, which in the case of B cells may have a higher affinity for the antigen, and both B and T cells that may have a lower threshold for activation. One model of immunological memory maintenance is that memory cells proliferate during primary antigen exposure, then persist in a non-proliferating state, or turnover homeostatically, in the blood, secondary lymphoid organs and bone marrow until secondary antigen exposure. An alternative model is that memory cells are not long-lived, but that they divide and replenish themselves to maintain the memory cell population long-term [235] (review [210, 244, 398]). This second model may or may not require the persistence of antigen in order to retain the memory cell pool.

Requirements for antigen persistence in order to maintain immunological memory are controversial [210, 248, 399]. Zinkernagel [210] argues that immunological memory – i.e. a faster response, is antigen-independent, but that protective immunity is antigen-dependent. Antigen may be maintained on follicular dendritic cells in secondary lymphoid

organs for weeks after the live infection has been eliminated, however it has been argued that antigen does not persist after the end of an infection (review [192]). If this is the case then establishing the length of infection is important in order to allow us to study whether longevity of immune responses is due to long-lived memory, concomitant immunity or to continuous turnover of antigen-experienced cells.

Arguably, as long as immunological memory is maintained, the method of its maintenance is less important. Determining the presence of residual antigen after pathogen clearance is difficult, the presence of small amounts of antigen somewhere in the body can rarely be ruled out, and the question of persistence of antigen that is not live parasites has not been addressed here. Nevertheless, if immune responses wane soon after the clearance of chronic infection, a case can be made for the requirement of parasite persistence. This may be the appropriate model for human malaria infections, as immune responses drop at the end of the transmission season [177, 305, 311, 313, 400]. In the C57BL/6 mouse model, antibody titres drop between 1 and 2 months after infection corresponding with the clearance of patent infection. There is, however, no obvious decline in antibody titres, either to whole parasite extract or to a defined malarial antigen, 3 months after infection when the subpatent parasitaemia is cleared [396]. This may indicate that memory B cells and long-lived plasma cells develop during the chronic infection, and will be the subject of further investigation.

Chapter 4

Architectural changes and cellular migration in the spleen during acute malaria infection

Introduction

The spleen in malaria

The spleen has a central role in the immune response to malaria and splenomegaly, although not unique to malaria infection, is nevertheless one of the most striking features of the malaria infection. Within the first week of infection it increases in size several fold, both in humans and mice [184, 345, 401-403], and in mice also darkens in colour, so that it is easily distinguishable from spleens of naïve mice (Figure 13). Although splenomegaly is maximal at peak parasitaemia and reduces thereafter, the spleen never returns to the size (or colour) it had pre-infection. Splenomegaly is due in part to increased erythropoiesis in mice [183, 184, 342-344], and influx of lymphocytes in human and mouse infections [344]. It is not caused by the parasite directly, but has been shown to be a T cell dependent response [404]. The extreme form of splenomegaly known as hyperreactive malarial splenomegaly has also been shown to be, at least partially, hereditary [403].

Splenectomy causes increased susceptibility to malaria in both humans [401] and rodents [258, 344, 351], with increased anaemia in splenectomised infected mice due to the greater erythropoietic role of the mouse spleen [30]. Injection of a naïve splenic cell suspension cannot compensate for splenectomy, suggesting a role for splenic microarchitecture or

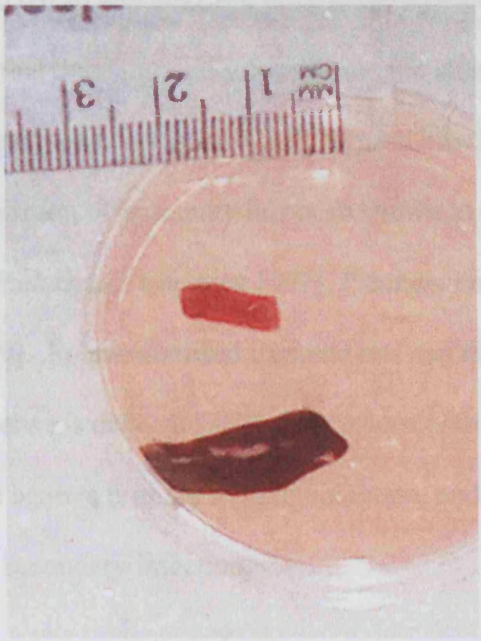


Figure 13. Photograph of naïve and malaria infected mouse spleens

Infected spleen is at day 20 post-infection

stromal cells in immune responses to malaria [258, 351]. Splenectomy does not, however, affect the development of anti-sporozoite immunity in response to immunisation [17]. The importance of the spleen in responses to primary infection may also have a genetic component, as D2 mice showed no effect of splenectomy on primary *P.yoelii* infection, whereas splenectomised C57BL/6 and BALB/c mice suffered more severe infections [405]. Some authors have shown that once immunity to erythrocytic stages has been acquired it may be maintained in the absence of the spleen in *P.berghei* infection [406], however splenectomy after the acquisition of immunity has been shown to reduce immunity in *P.vinckei* infection [258], *P.chabaudi* infection [407], *P.berghei* infection [408] and *P.falciparum* infection [409]. Splenectomised immune rats can clear a secondary infection, although the antibody response is delayed [369]. The spleen is therefore critical for an effective immune response against primary malaria infection, and still important for immune responses against secondary infection.

Splenic microarchitecture

Analysis of splenic structure in malaria infection shows large changes in both humans [348] and mice, particularly around peak parasitaemia [345-347]. These changes are only temporary however, and splenic structure returns to normal by day 60 [344, 345, 395]. Despite these changes, germinal centres still form and large numbers of plasma cells can be seen at the peak of infection [346]. It is not known however, whether these are part of a non-specific or malaria-specific immune response. It is also not known whether they remain in the spleen as short-lived plasma cells, or migrate to the bone marrow to become long-lived plasma cells.

In *P.chabaudi*, it has been observed that marginal metallophilic macrophages (MMM) and marginal zone B cells (MZ B cells) are lost from the marginal zone during the course of infection. MZ B cells have been shown to remain in the spleen [345, 346] and gradually return to the marginal zone [346], although their numbers remain reduced up to 120 days post-infection (R. Stephens, personal communication). Marginal zone macrophages (MZM) are also lost from the MZ in a *Leishmania donovani* infection [172] and after LPS treatment [349].

Macrophage depletion by administration of silica renders mice more susceptible to *P.chabaudi* malaria [410], and the effects of loss of macrophages from the MZ have been described in several models of systemic infectious disease. Depletion of MZM and MMM with low dose clodronate liposomes renders mice more susceptible to *Listeria monocytogenes* infection [411] and lymphocytic choriomeningitis virus (LCMV) infection [412], while high dose treatment also affects immunity to re-infection [413]. MZM have been shown to be required for the retention of MZ B cells in the MZ [414]. Work in other laboratories has demonstrated further changes in splenic microarchitecture, involving altered location of dendritic cells [415], macrophages [416] and formation of barrier cells [184, 344, 347].

In order to understand what happens in the spleen during acute infection, and to understand the differences between changes in mouse and human spleens, we need to thoroughly characterise what changes occur in the spleen, when during infection they occur, what causes these changes and whether they differ with repeated exposure to the parasite. This chapter shows a histological examination of spleens for days 0-10 of the acute infection, with two later timepoints at days 14 and 20. The following parameters were investigated

with respect to extent of changes, chronological order and the time of infection when alterations become visible:

1) Location of parasites in the spleen
2) Segregation of T cells zones and B cell follicles
3) Distribution of dendritic cells, $CD8^+$ and $CD8^-$ subsets and plasmacytoid dendritic cells, and their migration within the spleen during the acute infection
4) Distribution of red pulp macrophages
5) Alterations in the structure and composition of the marginal zone
6) Alterations in fibroblasts, reticular cells and expression of cell surface and adhesion molecules
7) Formation of germinal centres and plasma cells
8) Possible mechanism of alterations of the splenic microarchitecture

Results

In order to determine changes in the microarchitecture of the spleen during the first ten days of infection and to compare them to later stages of infection, spleens were taken from *P.chabaudi* infected mice as shown in Figure 14.

Controls

One potential problem of multi-colour staining of spleen sections with the available antibodies is cross-reactivity of secondary antibodies or reagents. Therefore, all second-stage reagents were tested alone, in combination and together with isotype controls for the primary antibodies. Representative isotype controls from naïve and infected mice for all experiments are shown in Figure 15. Cross-reaction of the anti-rat IgM Alexa 488 antibody with mouse IgM could not be eliminated by pre-incubating sections with anti-FcR or with unlabelled anti-mouse IgM, therefore MZM staining with the ER-TR9 antibody could not be transferred to immunofluorescence. All other secondary antibodies used did not cross-react with mouse immunoglobulins and no staining was seen with primary antibody isotype controls and appropriate secondary antibodies.

Co-staining and colour overlap

Some cell surface molecules are not expressed on the same cells, however confocal microscopy at low magnification (x 30.4) cannot always distinguish between the expression of such molecules on the same cell and their expression on closely neighbouring cells. In this situation, true co-staining can be distinguished by examination of images at higher magnification (Figure 16, Figure 17), and can be objectively determined by colour percentage analysis of the degree of colour overlap at low and high magnification.

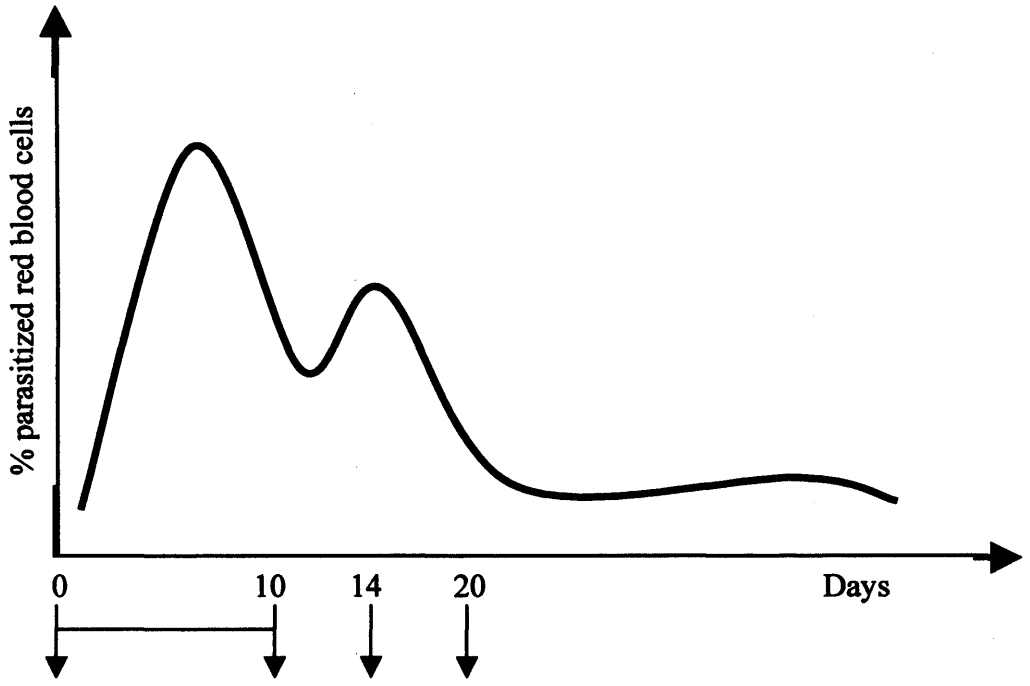
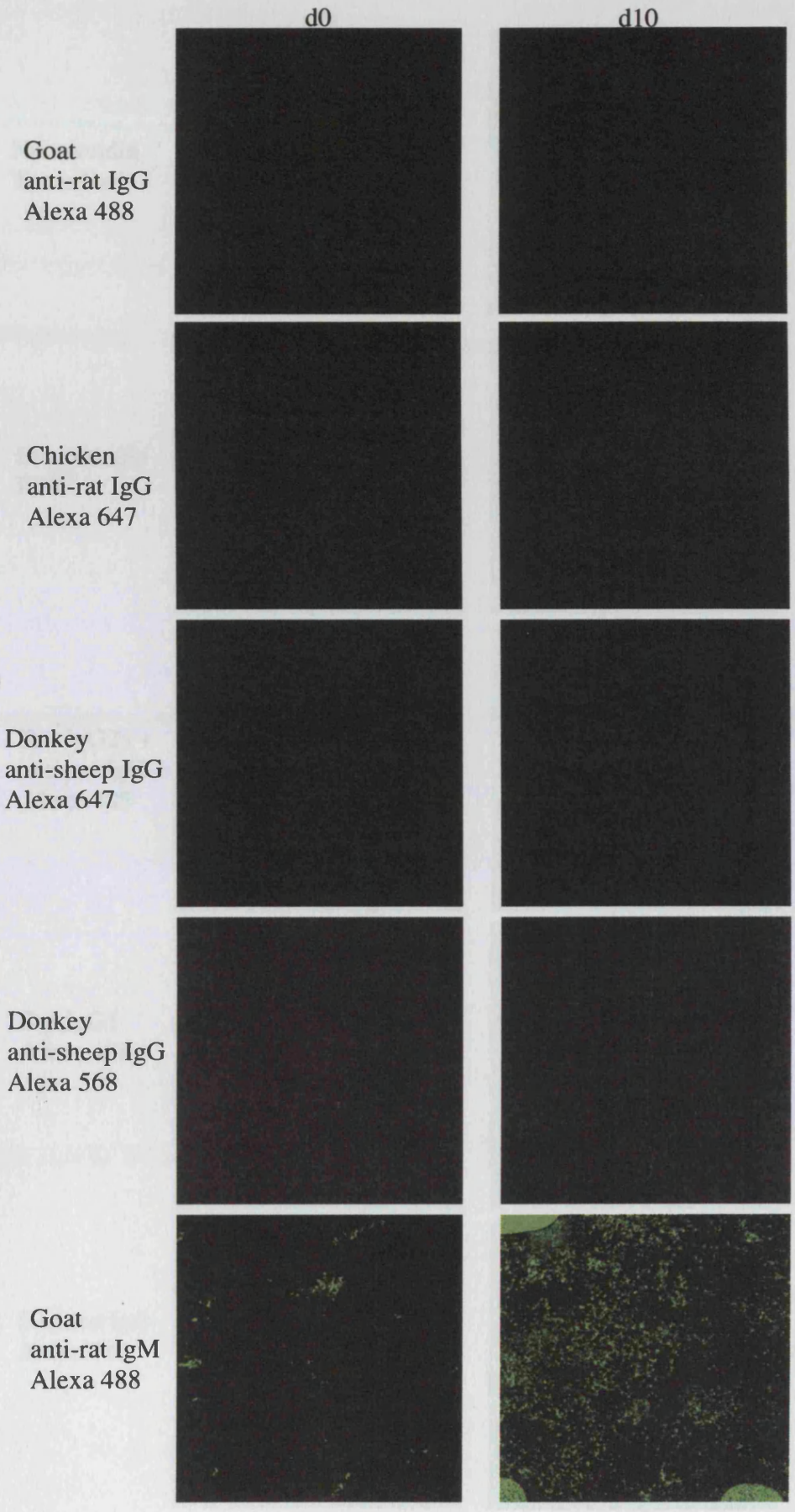
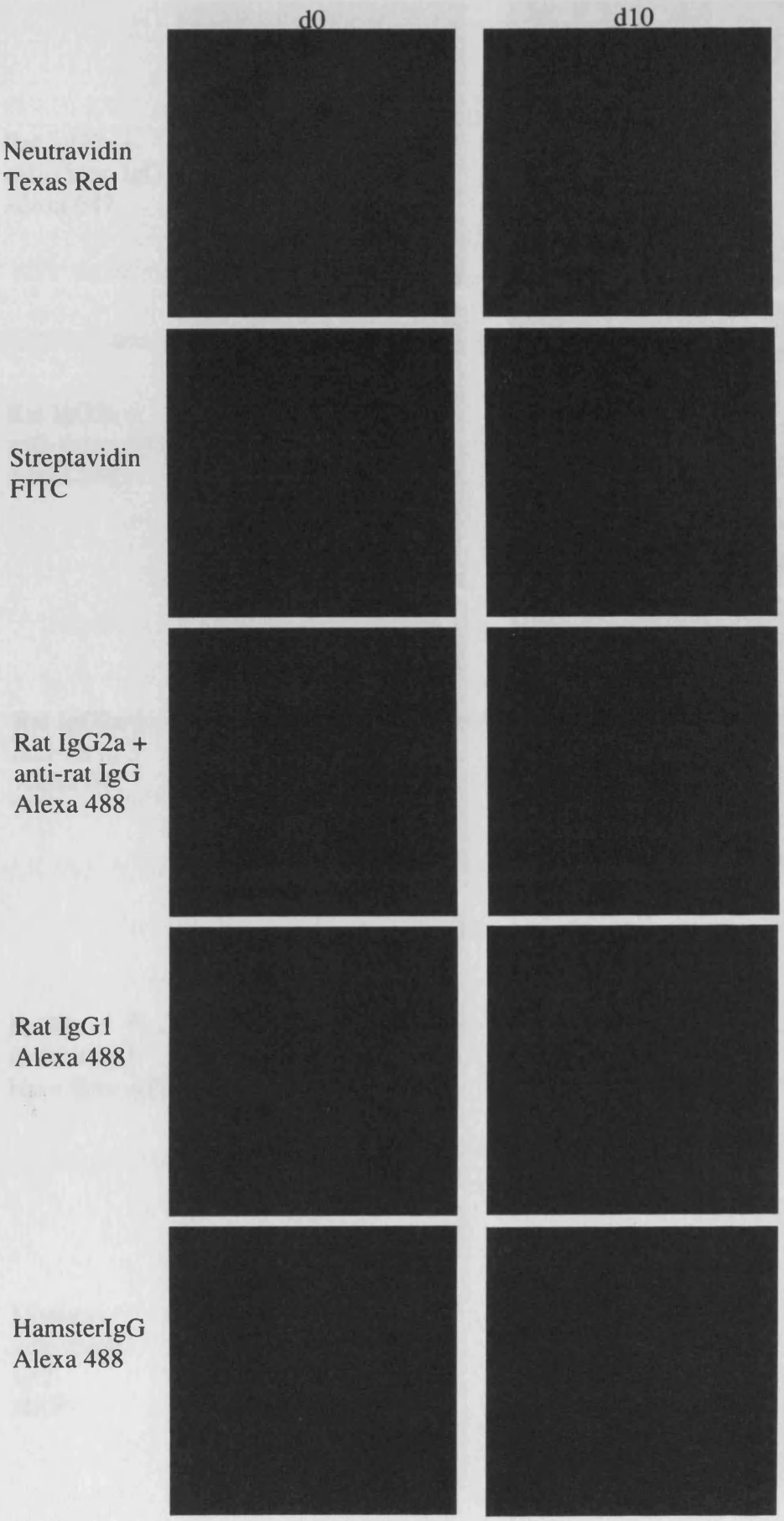


Figure 14. Experimental plan of histological analysis of spleens during the acute malaria infection

Figure 15. Isotype controls for immunohistology

Black spots on immunocytochemistry sections * (rabbit anti-rat biotin & donkey anti-sheep HRP) are Indian Ink taken up by marginal zone macrophages


d0
d10
Goat
anti-rat IgG
Alexa 488
Chicken
anti-rat IgG
Alexa 647
Donkey
anti-sheep IgG
Alexa 647
Donkey
anti-sheep IgG
Alexa 568
Goat
anti-rat IgM
Alexa 488


d0
d10
Neutravidin
Texas Red
Streptavidin
FITC
Rat IgG2a +
anti-rat IgG
Alexa 488
Rat IgG1
Alexa 488
HamsterIgG
Alexa 488


d0
d10
Rat IgG2a +
anti-sheep IgG
Alexa 647
Rat IgG2a +
anti-sheep IgG
Alexa 568
Rat IgG2a +
anti-rat IgG
Alexa 647
Rabbit * a
anti-rat IgG
bio + SAv AP
Donkey *
anti-sheep
IgG
HRP

Figure 16. False co-staining by expression of cell surface molecules on closely neighbouring cells

A) Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD3 & goat anti-rat IgG Alexa 488 (green) for T cells.

B) Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse F4/80 & goat anti-rat IgG Alexa 488 (green) for macrophages.

At higher magnification there is little co-localisation of the two stains. False double staining can only be seen at one side of a cell where it is next to one stained with the other marker and is seen at the edge of cell populations. Day post-infection of spleen sections is indicated to the left of the images.

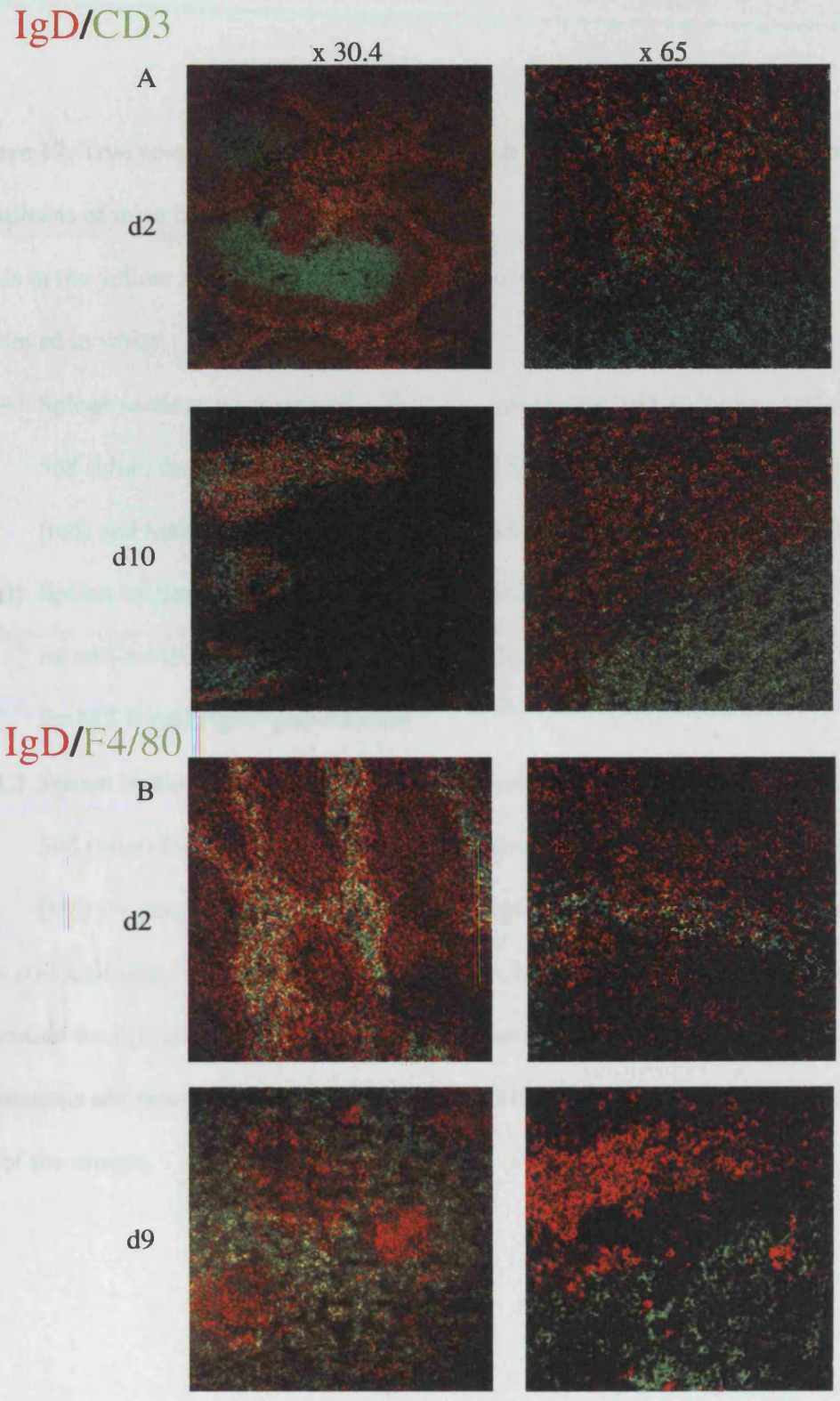

IgD/CD3
x 30.4
x 65
A
d2
d10
IgD/F4/80
B
d2
d9

Figure 17. True co-staining of $CD8^+CD11c^+$ dendritic cells, IgM^+ and IgG^+ plasma cells in the spleens of mice infected with *P.chabaudi*

Pixels in the yellow spectrum, as selected by the colour picker plugin for ImageJ, are overlayed in white

A) Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (blue) for B cell follicles, rat anti-mouse CD8 & chicken anti-rat Alexa 647 (red) and hamster anti-mouse CD11c Alexa 488 (green) for dendritic cells.

B) Spleen sections were stained with sheep anti-mouse IgD (blue) for B cell follicles, rat anti-mouse CD138 (red) for plasma cells and rat anti-mouse IgM FITC (green) for MZ B cells/IgM^+ plasma cells

C) Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (blue) for B cell follicles, rat anti-mouse CD138 & chicken anti-rat Alexa 647 (red) for plasma cells and rat anti-mouse IgG FITC (green) for IgG^+ plasma cells

True co-localisation of antibodies to CD8/CD11c, IgM/CD138 and IgG/CD138 can be seen all around the cell and in the centre of single stained populations, not just where two populations are next to each other. Day post-infection of spleen sections is indicated to the left of the images.

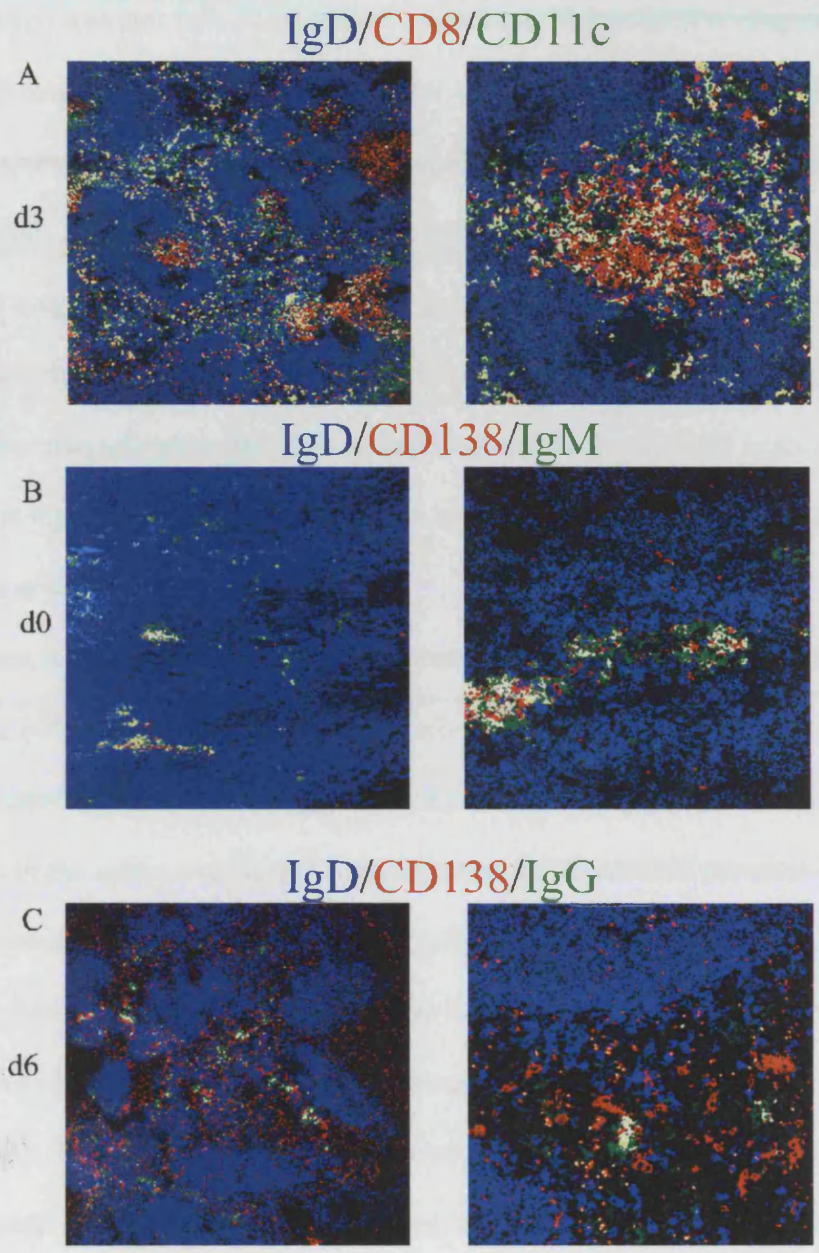

IgD/CD8/CD11c
A
d3
IgD/CD138/IgM
B
d0
IgD/CD138/IgG
C
d6

Table 7 shows a colour analysis of the images shown in Figures 16 & 17. The percentage of the image area that falls in the yellow spectrum is higher for low magnification images than high magnification images from Figure 16. This indicates that the colour overlap is due to expression of different cell surface molecules on closely neighbouring cells, which is as expected. By comparison, the colour overlap percentage is higher at high magnification than low magnification for images from Figure 17, indicating that these cells co-express both molecules, also as expected. Higher levels of colour overlap at lower magnification than higher magnification therefore indicate false co-staining, while higher levels of colour overlap at higher magnification than lower magnification indicate true co-staining.

Location of parasites

The spleen is the primary site of contact between antigens in the blood and lymphocytes in the white pulp of the spleen. It is particularly relevant for malaria infection, where the disease-causing phase of the life-cycle is an infection of erythrocytes. The early location of parasites in the spleen was identified by injecting CFSE labelled parasites into the mouse and harvesting spleens 1 and 4 hours post infection. Confocal microscopy of spleen sections shows the majority of the parasites in the red pulp and marginal zone, however some have already penetrated into the white pulp as little as 1 hour after injection (Figure 18 A & B). The location of parasites in relation to $CD11c^+$ dendritic cells was also determined. Parasites were seen in close proximity to dendritic cells, however there was little co-localisation of CFSE and CD11c, giving no indication that dendritic cells have taken up parasites at this early timepoint (Figure 18 C & D). Further work would be required to determine the time and location of the initial uptake of parasites by dendritic cells.

Table 7. Incidence of co-staining in images taken at low and high magnification

Stain	Day	Magnification (x)	% co-staining
IgD/CD3	2	30.4	0.59
IgD/CD3	2	65	0.36
IgD/CD3	10	30.4	0.56
IgD/CD3	10	65	0.26
IgD/F4/80	2	30.4	4.56
IgD/F4/80	2	65	1.27
IgD/F4/80	9	30.4	2.69
IgD/F4/80	9	65	1.63
CD8/CD11c	3	30.4	2.98
CD8/CD11c	3	65	4.78
CD138/IgM	0	30.4	0.25
CD138/IgM	0	65	1.58
CD138/IgG	0	30.4	0.39
CD138/IgG	0	65	0.51

Co-staining was measured using the colour picker plugin (Daan Zhu, NIMR) for ImageJ.

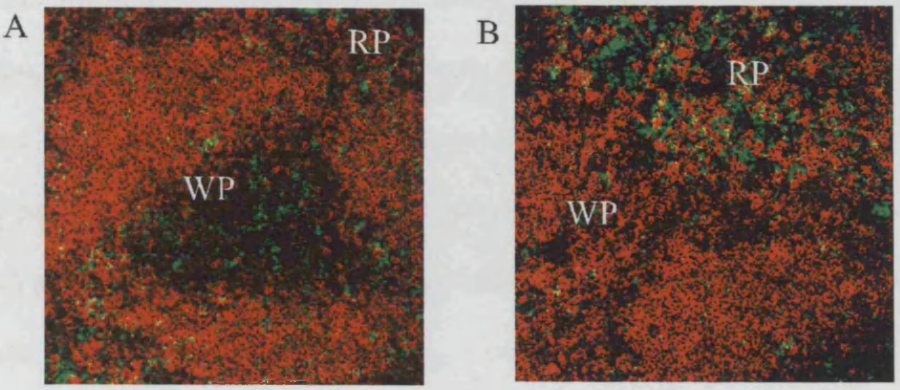
Figure 18. Location of parasitised erythrocytes within the spleen 1 hour after infection with 2 x 10^7 *P.chabaudi* pRBC

A and B) Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (red) for B cell follicles. pRBC were labelled with CFSE (green) and had been injected i.v. into naïve mice 1 hour previously

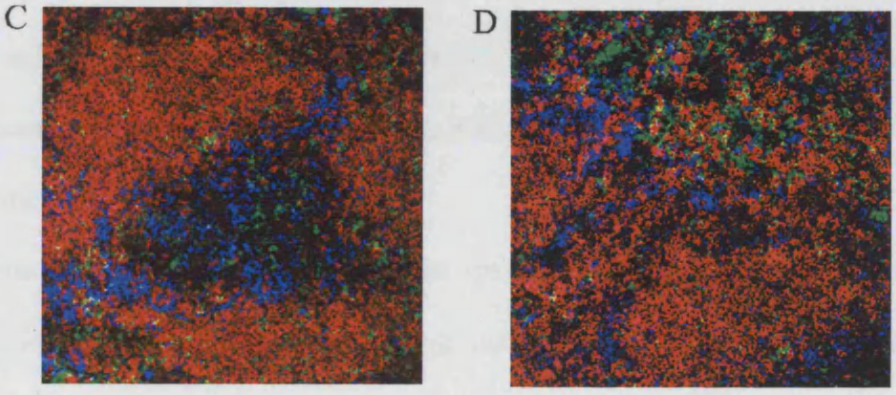
C and D) Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles, and hamster anti-mouse CD11c biotin & neutravidin Texas Red for dendritic cells (blue). pRBC were labelled with CFSE (green) and injected i.v. into naïve mice.

Images shown are representative of the whole spleen and of two mice. A and C show mostly white pulp (WP), B and D show white pulp and red pulp (RP). Magnification x 65

IgD/CFSE



IgD/CFSE/CD11c



Segregation of B and T cell zones

Merging of T and B cell areas during the acute malaria infection is shown in Figure 19. T and B cell areas can be clearly defined in the naïve mouse with antibodies to CD3 (T cells) and IgD (naïve follicular B cells). These areas remain well segregated until day 5 of infection, when increasing numbers of T cells can be observed in the red pulp. Segregation of B and T cells continues to lessen as the parasitaemia increases, reaching a minimum at day 10 post infection, at the time of peak parasitaemia. At day 14 post infection B cell follicles are thinner and more ring-like, as follicular B cells have been pushed out to form a follicular mantle around germinal centres. T cells can be seen clustered next to follicles but not inside them, and both T and B cells are mostly absent from the red pulp. As the parasitaemia is cleared, T and B cell areas re-form, although they have still not regained their original locations by day 20. The images shown in Figure 19 are representative of the whole spleen, and of three mice. These results confirm what has been previously demonstrated by other investigators [345, 346].

Dendritic cells

The cells that initiate the immune response are the antigen-presenting cells, particularly the conventional dendritic cells, which express high levels of CD11c [55], but also plasmacytoid dendritic cells, which are capable of antigen presentation to naïve T cells [67] (though more efficient at antigen presentation to antigen-experienced cells) and express low to intermediate levels of CD11c [49, 64, 67, 68]. In naïve mice, $CD11c^+$ cells are present in the red pulp, marginal zone and white pulp. Increasing numbers of $CD11c^+$ cells can be seen throughout the red pulp from day 5 of *P.chabaudi* infection, however by day 10 post-infection they have clustered in the red pulp close to the remaining B cell follicles, where

Figure 19. Reduced segregation of B and T cell areas in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD3 & goat anti-rat IgG Alexa 488 (green) for T cells. Images shown are representative of the whole spleen and of three mice. Areas of red pulp (RP) and white pulp (WP) are indicated. Magnification x 30.4

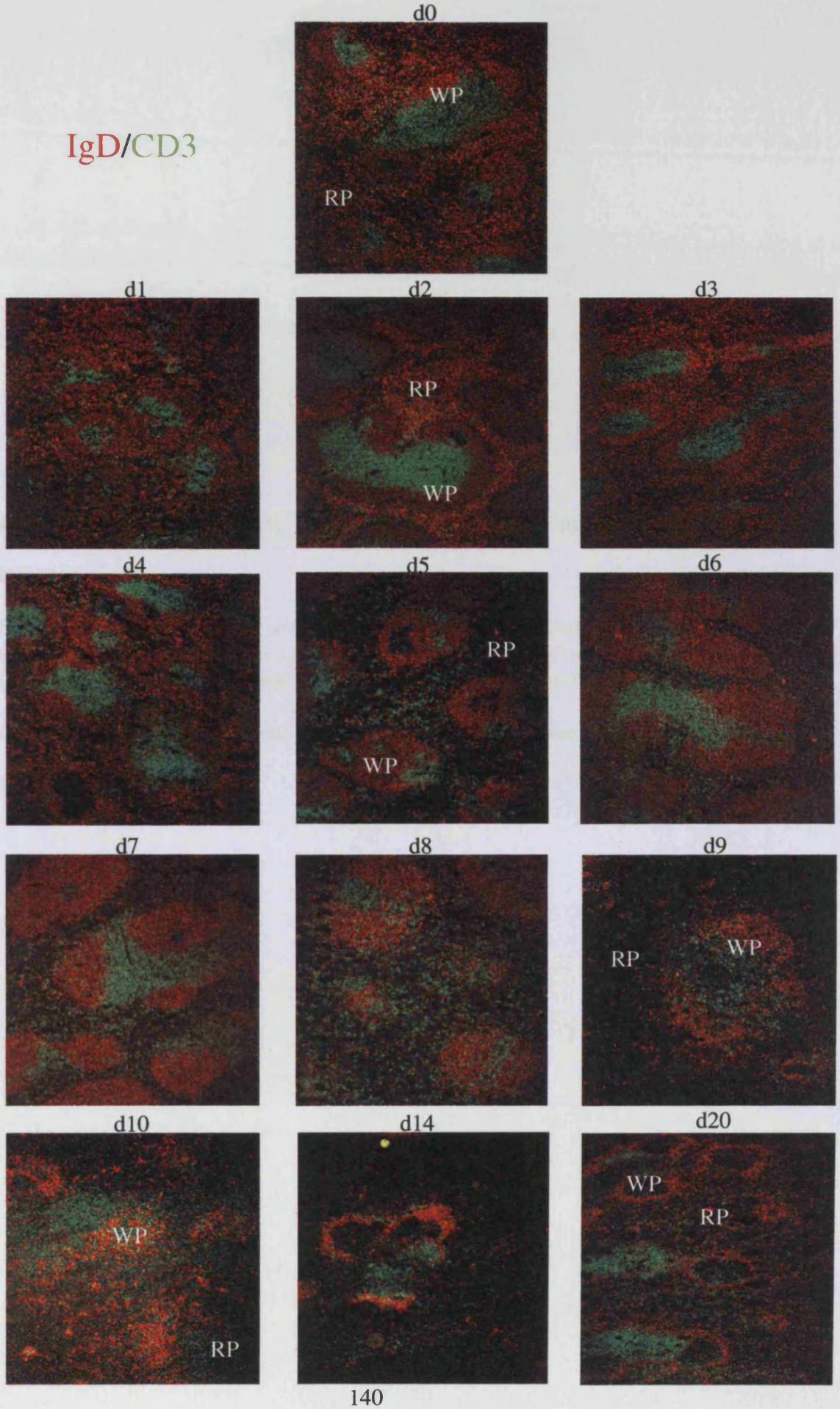
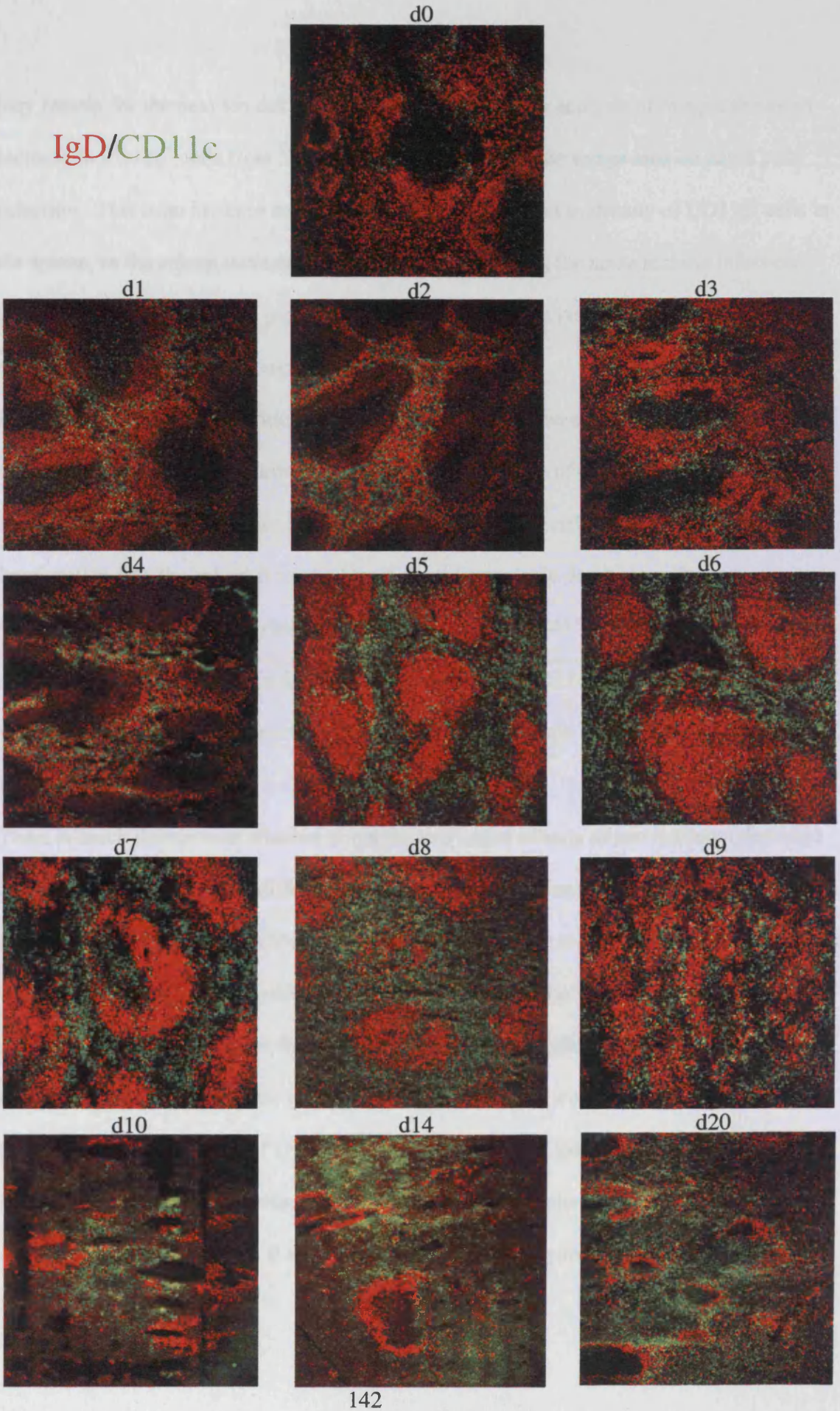

IgD/CD3
d0
WP
RP
d1
d2
RP
WP
d3
d4
d5
RP
WP
d6
d7
d8
d9
RP
WP
d10
WP
RP
d14
d20
WP
RP

Figure 20. Location of $CD11c^+$ dendritic cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and hamster anti-mouse CD11c Alexa 488 (green) for dendritic cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4


IgD/CD11c
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

they remain for the next ten days (Figure 20). Colour picker analysis of images shows an increase in $CD11c^+$ cells from 5% in naïve mice to 71% of the image area on day 8 post-infection. This is an increase not only in numbers of cells,but in density of $CD11c^+$ cells in the spleen, as the spleen increases several fold in size during the acute malaria infection (Figure 13), therefore colour picker analysis of images at the peak of parasitaemia under-represents cell numbers in comparison to naïve mice.

Increasing numbers of dendritic cells in the spleen can also be seen by flow cytometry from day 5 post infection, with numbers decreasing after the peak of parasitaemia [61] (Figure 21A). It is not known whether this increase is a result of dendritic cell proliferation or immigration into the spleen from the blood. Resident splenic dendritic cells are $CD11c^{+/high}$, whereas monocytes in the blood are $CD11c^{-/int}$ [417]. The majority of the cells responsible for the increase in dendritic cell numbers are $CD11c^{int}$ cells (Figure 21B), which may suggest that the increase in dendritic cells is mostly due to recruitment of cells from the blood.

There is much debate over whether or not the two major subsets of conventional dendritic cells, $CD8^+$ and $CD8^-$, have different functions. Spleen sections were therefore co-stained with antibodies to CD8 and CD11c to determine whether the two populations have different locations, possibly indicating different roles in the acute *P.chabaudi* infection. In the naïve mouse, $CD8^-CD11c^+$ DCs are found in the red pulp and marginal zone, while $CD8^+CD11c^+$ DCs are found primarily in the white pulp (Figure 22). In infected mice, there is already an increase in the proportions of $CD8^-$ $CD11c^+$ DC relative to $CD8^+CD11c^+$ DC by day 5 post infection (Figure 21A). By this time there are increased numbers of $CD8^-CD11c^+$ DC in red pulp (Figure 22). As the B and T cell areas become indistinguishable both DC

Figure 21. Increasing numbers of dendritic numbers in the spleen during acute *P.chabaudi* infection determined by flow cytometry analysis

A) Total $CD11c^{+}$ cells

B) $CD11c^{intermediate}$ cells

C) $CD11c^{high}$ cells

A-M. Sponaas, E. Cadman, C. Voisine, V. Harrison, A. Boonstra, A. O'Garra & J. Langhorne, J Exp Med 2006 203(6) 1427-33

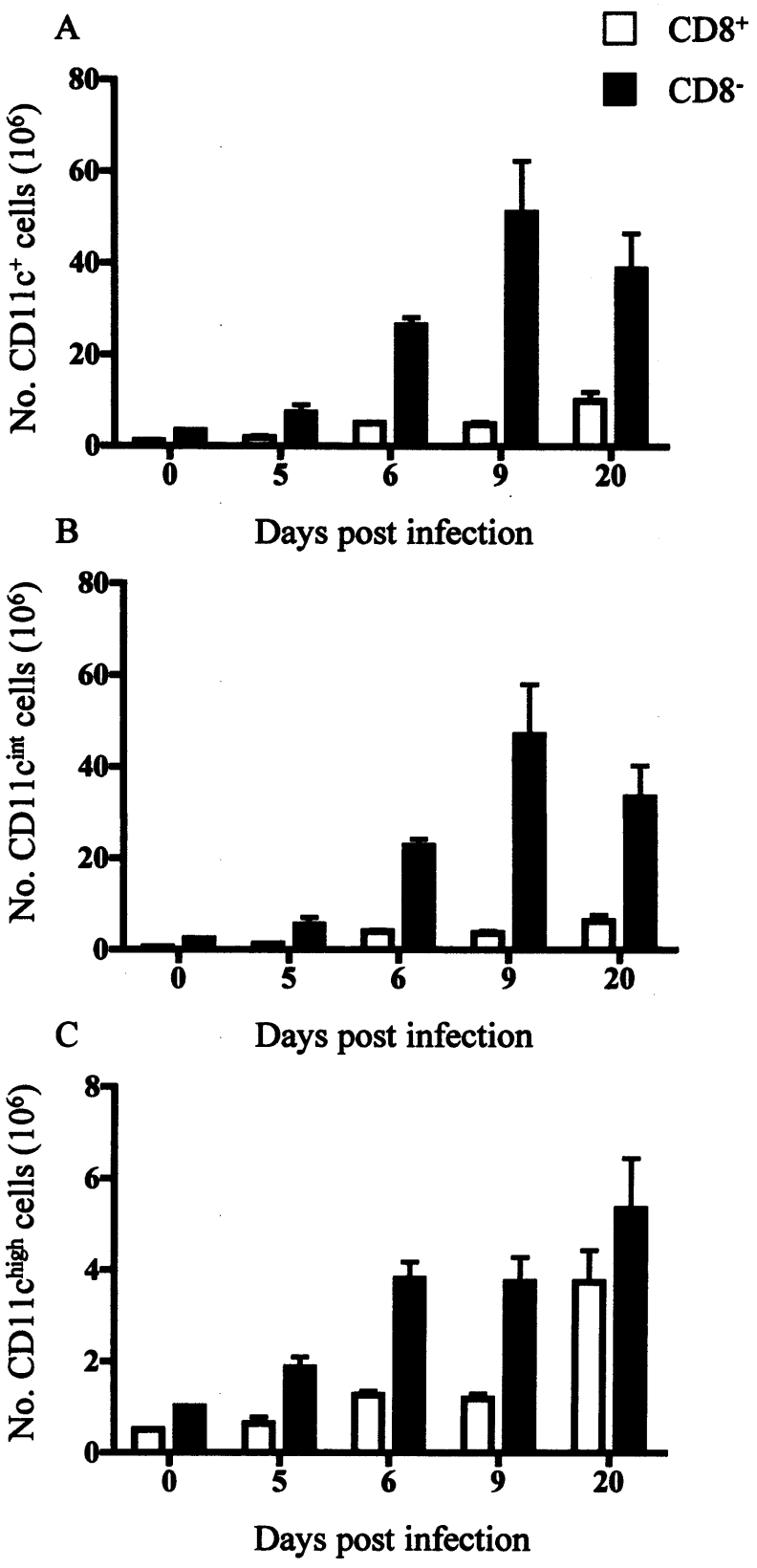
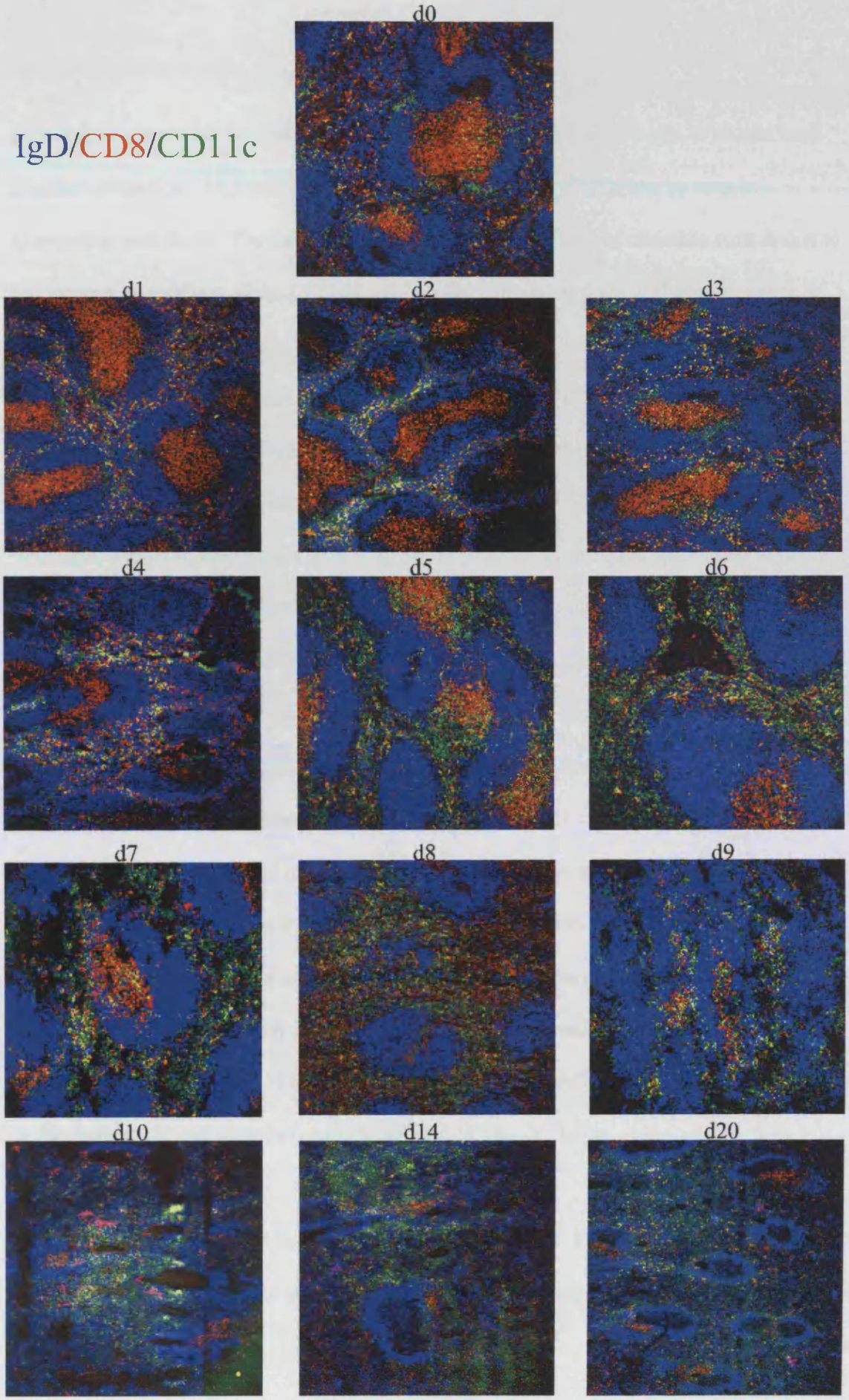

A
CD8+
CD8-
No. CD11c+ cells (10^6)
80
60
40
20
0
0
5
6
9
20
Days post infection
B
No. CD11c^int cells (10^6)
80
60
40
20
0
0
5
6
9
20
Days post infection
C
No. CD11c^high cells (10^6)
8
6
4
2
0
0
5
6
9
20
Days post infection

Figure 22. Location of $CD8^+$ and $CD8^-$ conventional dendritic cell subsets in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (blue) for B cell follicles, hamster anti-mouse CD11c Alexa 488 (green) for $CD8^-$ dendritic cells and rat anti-mouse CD8 & chicken anti-rat Alexa 647 for $CD8^+$ T cells (red) and $CD8^+$ dendritic cells (yellow). Images shown are representative of the whole spleen and of three mice. Magnification x 30.4


IgD/CD8/CD11c
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

populations are spread throughout the spleen, then as B and T cells begin to cluster back together around day 14, both $CD8^-CD11c^+$ and $CD8^+CD11c^+$ DCs can be seen re-associating with them. The majority of the increase in numbers of dendritic cells is due to an increase in numbers of the $CD8^-CD11c^+$ subsets, however there is also an increase in number of $CD8^+CD11c^+$ DCs. Whether this increase in DC numbers is due to an influx of cells from the blood or to proliferation of resident splenic DCs has not been determined. Numbers of $CD8^+CD11c^+$ DCs peak later around day 20, whereas $CD8^-CD11c^+$ DC numbers peak around the peak of parasitaemia [61] (Figure 21A). Colour picker analysis of images shows similar proportions of $CD8^+$ DCs (3.44%) and $CD8^-$ DCs (3%) in naïve mice, increasing to 5.6% $CD8^+$ DCs and 14% $CD8^-$ DCs by day 5 post-infection, in agreement with flow cytometry data. $CD8^-$ DCs further increase to 16.4% on day 8 and 21.7% on day 14 post-infection. The proportion of $CD8^+$ DCs drops slightly to 4.5% on day 8 and 3.4% on day 14, however these numbers refer to the density of $CD8^+$ DCs in the images, rather than the absolute number of cells.

Plasmacytoid DCs are found in the red and white pulp in the spleens of naïve mice, but not in the marginal zone (Figure 23) [80]. They remain mostly in the red pulp during a malaria infection and do not migrate into the white pulp, although they increase in number up to day 20 post-infection (Figure 24E). Prior to the peak of parasitaemia, days 5-7 post infection, they can be seen in the marginal zone as well, moving back out into the red pulp at the peak of parasitaemia between days 8 and 10 post-infection.

Macrophages

Macrophages are important in malaria infection for removal of parasites, and macrophage depletion renders mice more susceptible to malaria infection [410]. Red pulp macrophages

Figure 23. Location of plasmacytoid dendritic cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse 120G8 & goat anti-rat IgG Alexa 488 (green) for plasmacytoid dendritic cells. Images shown are representative of the whole spleen and of three mice. Magnification x 65

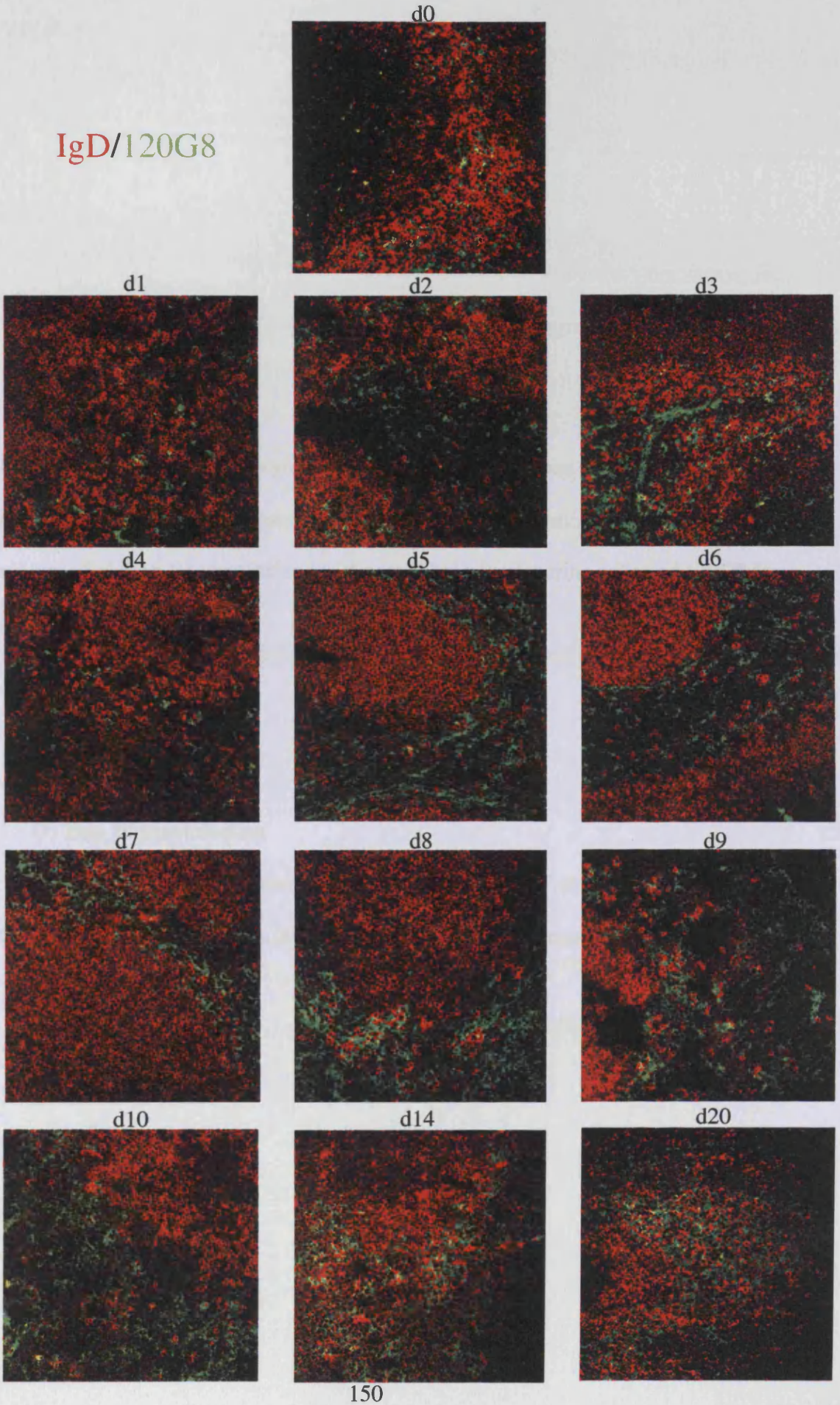

IgD/120G8
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Figure 24. Profile of plasmacytoid dendritic cells in the spleen during acute *P.chabaudi* infection. Spleen cell suspensions were made by liberase treating naïve and infected spleens. Splenocytes were stained with anti-CD11c for dendritic cells and 120G8 for pDCs.

A) Naïve mouse

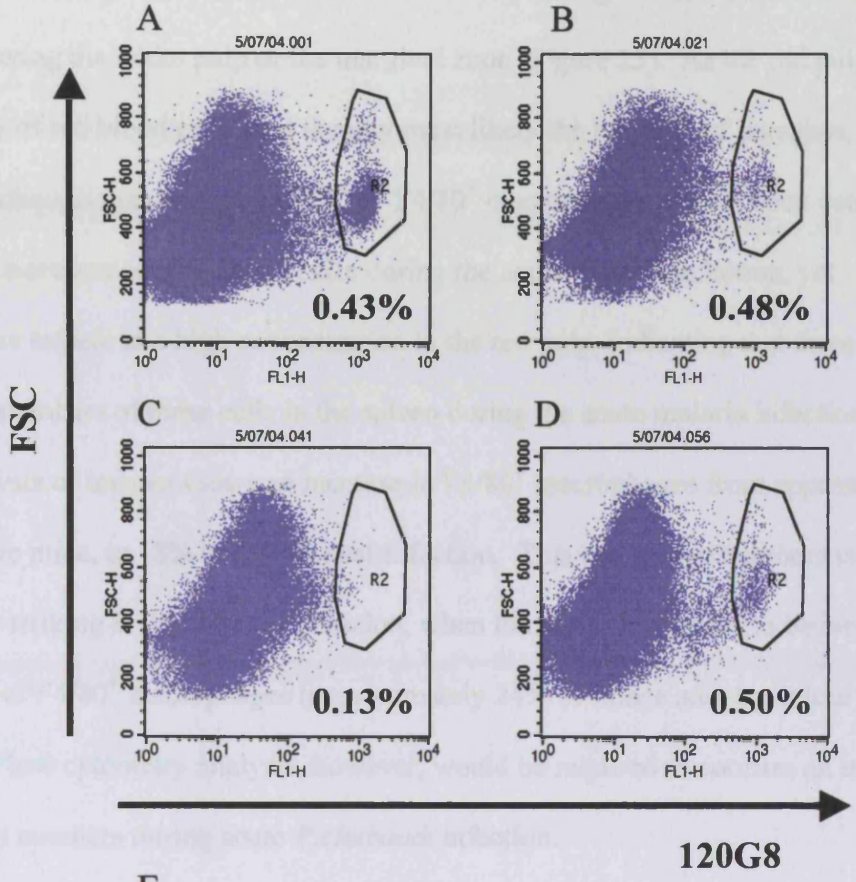
B) Day 5 post infection

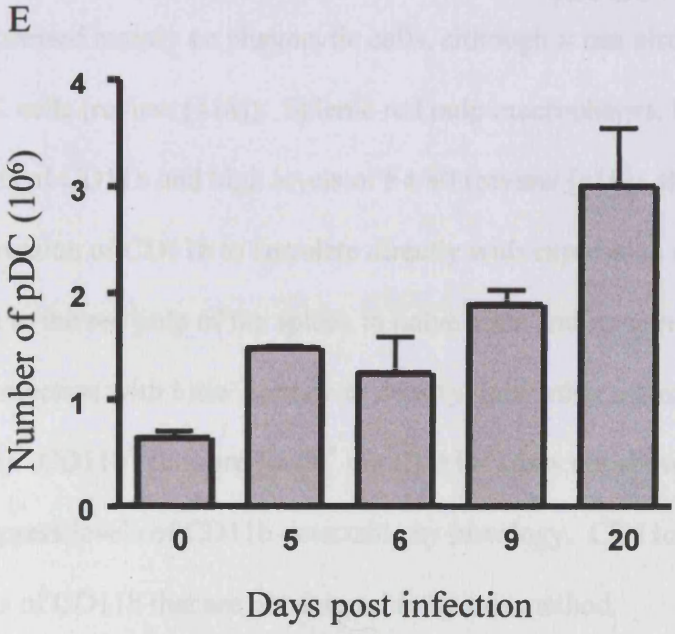
C) Day 9 post infection

D) Day 20 post infection

E) Changes in pDC number in the spleen during acute *P.chabaudi* infection

C. Voisine, E. Cadman, A-M. Sponaas & J. Langhorne, in preparation


A
5/07/04.001
R2
0.43%
B
5/07/04.021
R2
0.48%
C
5/07/04.041
R2
0.13%
D
5/07/04.056
R2
0.50%
FSC-H
FL1-H
FSC
120G8


E
Number of pDC (10⁶)
0
1
2
3
4
0
5
6
9
20
Days post infection

are visualised with an antibody to the cell surface molecule F4/80. They are clearly visible solely in the red pulp in naïve mice, and remain there throughout the acute infection without entering the white pulp or the marginal zone (Figure 25). As the red pulp contains the majority of red blood cells, and thereby most likely the majority of parasites, this is expected, although the uptake of pRBC by F4/80$^+$ macrophages has not been determined. The spleen increases several-fold in size during the acute malaria infection, yet macrophages remain at a high concentration in the red pulp, indicating that there is an increase in numbers of these cells in the spleen during the acute malaria infection. Colour picker analysis of images shows an increase in F4/80$^+$ macrophages from approximately 11% in naïve mice, to 18% at day 10 post-infection. This increase in numbers is particularly striking at day 14 post-infection, when the red pulp appears to be largely constituted of F4/80$^+$ macrophages (approximately 24% of image area by colour picker analysis). Flow cytometry analysis, however, would be required to confirm an increase in macrophage numbers during acute *P.chabaudi* infection.

CD11b (Mac-1) is expressed mainly on phagocytic cells, although it can also be expressed on some B, T, and NK cells (review [418]). Splenic red pulp macrophages, however express only low levels of CD11b and high levels of F4/80 (review [419]), therefore we would not expect expression of CD11b to correlate directly with expression of F4/80. CD11b$^+$ cells are seen in the red pulp of the spleen in naïve mice and remain there throughout the acute infection with little increase in density, indicating a possible increase in numbers (Figure 26). CD11b$^+$ cells are F4/80$^+$ not CD11c$^+$ (data not shown), although not all F4/80$^+$ cells express levels of CD11b detectable by histology. CD11c$^+$ cells may also express low levels of CD11b that are not detectable by this method.

Figure 25. Location of F4/80^{+} macrophages in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse F4/80 & goat anti-rat IgG Alexa 488 (green) for red pulp macrophages. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4

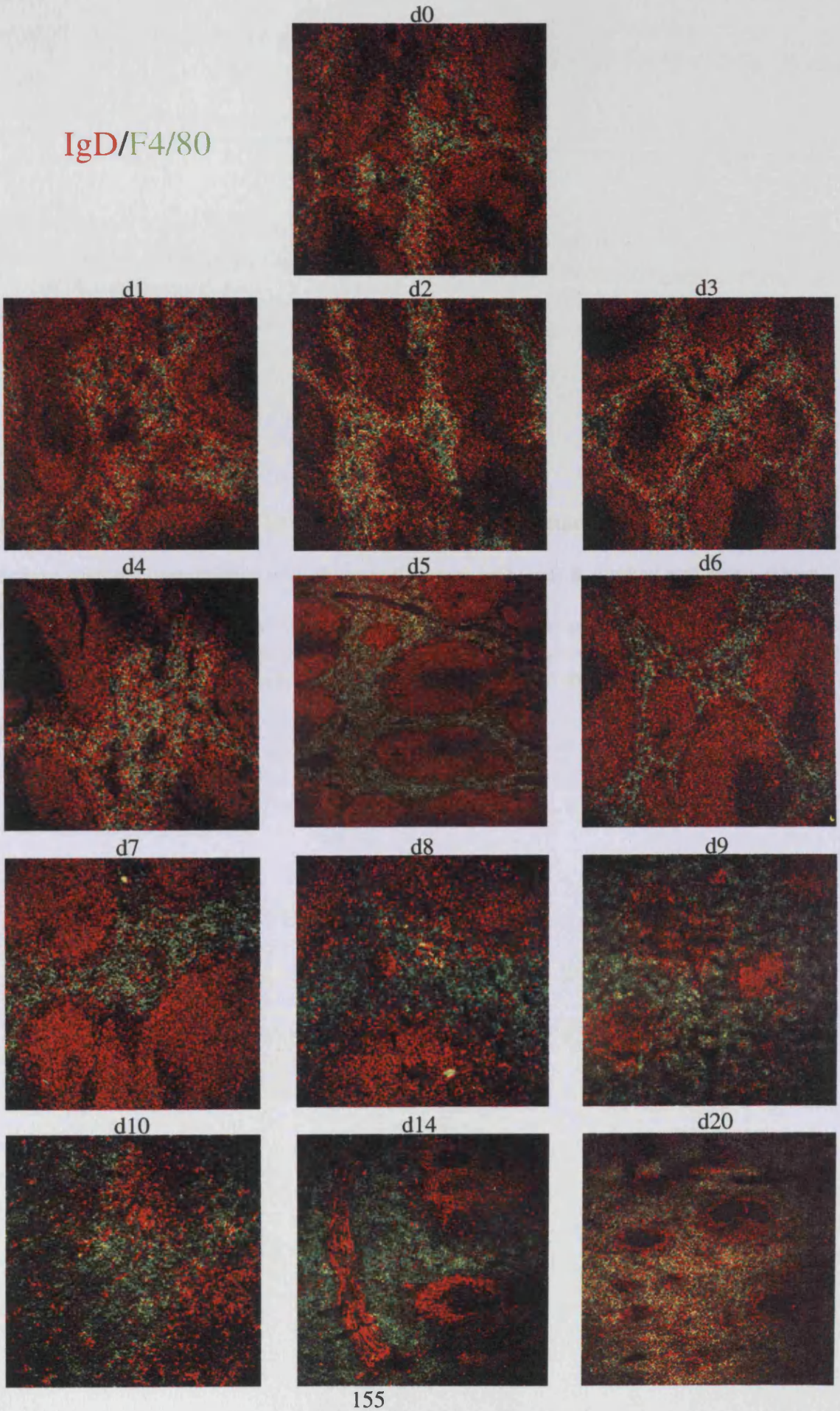
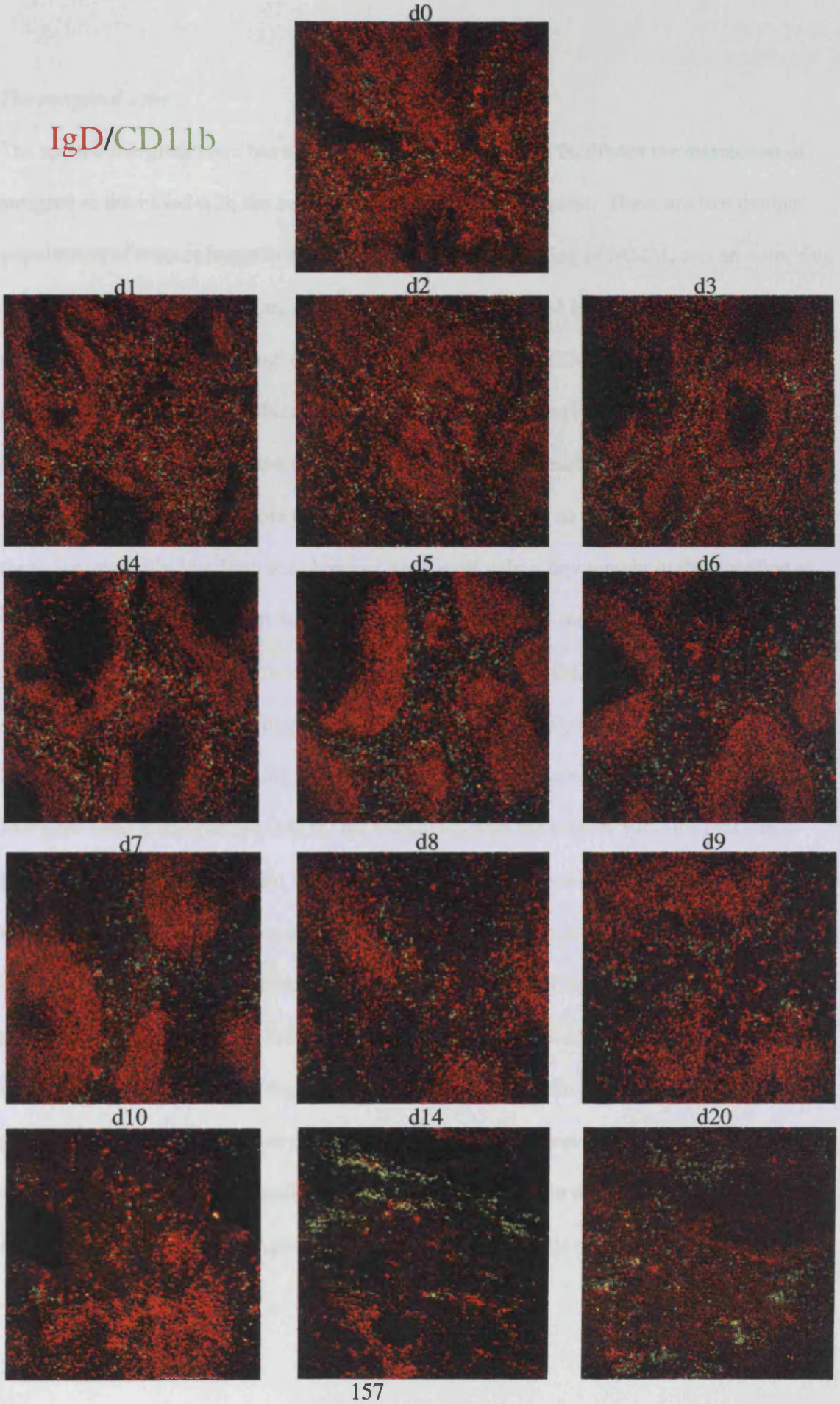

IgD/F4/80
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Figure 26. Location of CD11b$^+$ cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD (red) & donkey anti-sheep Alexa 647 for B cell follicles and rat anti-mouse CD11b & goat anti-rat IgG Alexa 488 (green) for CD11b$^+$ cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4


IgD/CD11b
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

The marginal zone

The splenic marginal zone has a specialised structure, which facilitates the interaction of antigens in the blood with the cells residing in the marginal zone. There are two distinct populations of macrophages in the marginal zone; an inner ring of MMM, and an outer ring of MZM [186]. MZ B cells are closely associated with MZM in the outer ring, and memory B cells are also thought to be located here [420]. MZM and MMM lie on either side of the marginal sinus, which is lined by endothelial cells (Figure 27) [186].

MMM are distinguished by their expression of CD169 (sialoadhesin/siglec-1) and form a complete ring around the white pulp in naïve mice, however as the infection progresses these are gradually lost from the marginal zone until only a few remain in this location at day 10 post infection, the rest being scattered throughout the red pulp (Figure 28A). Detection of another cell surface molecule expressed by MMM, MOMA-1, produces the same picture (Figure 28B), suggesting that these cells are truly being lost from this location and are not just downregulating expression of cell surface molecules.

Marginal zone macrophages (MZM) are visualised with the marker ER-TR9 (SIGNR1 [44]). They are clearly present in the marginal zone in naïve mice, but begin to be lost from this location by day 6, are almost entirely absent from this location by day 10 post-infection, and are instead scattered throughout the red pulp (Figure 29). An alternative method of visualising MZM is to inject mice intravenously with Indian ink, which is taken up selectively by MZM, one day prior to infection. MZM which have taken up carbon particles can be seen in the marginal zone in naïve mice, however these gradually disappear as the infection progresses, until they can no longer be seen in the marginal zone by day 10 post infection (Figure 29). Again this suggests that these cells are being completely lost

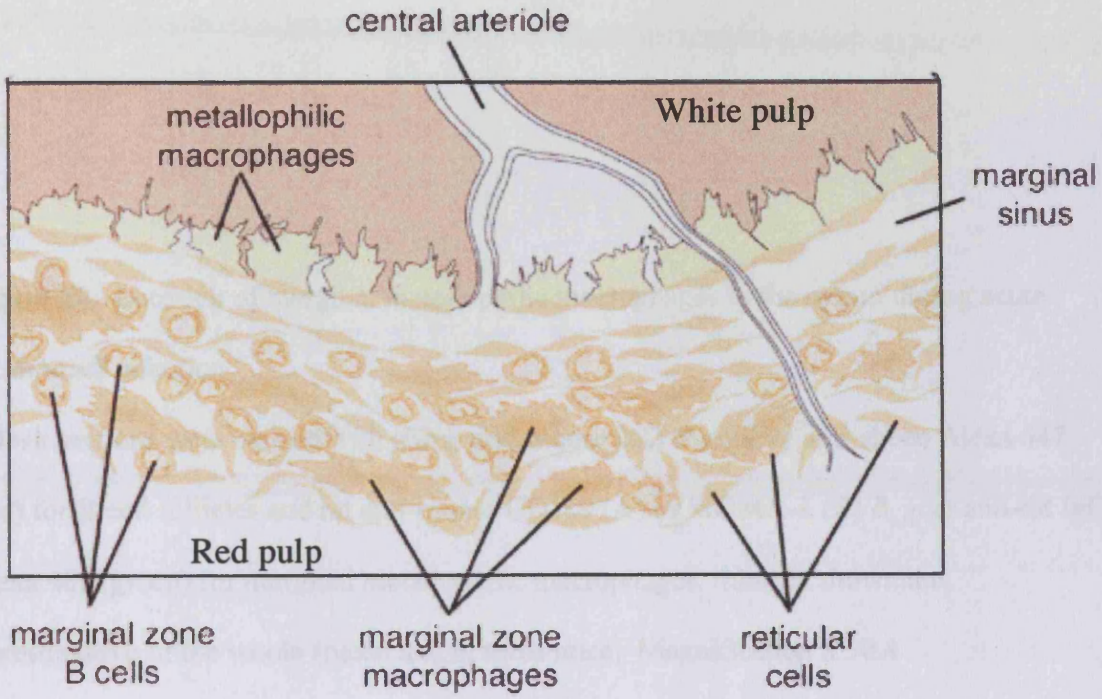


Figure 27. Structure of the marginal zone

Adapted from [186]

Figure 28. Location of marginal metallophilic macrophages in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD169 (A) or MOMA-1 (B) & goat anti-rat IgG Alexa 488 (green) for marginal metallophilic macrophages. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4

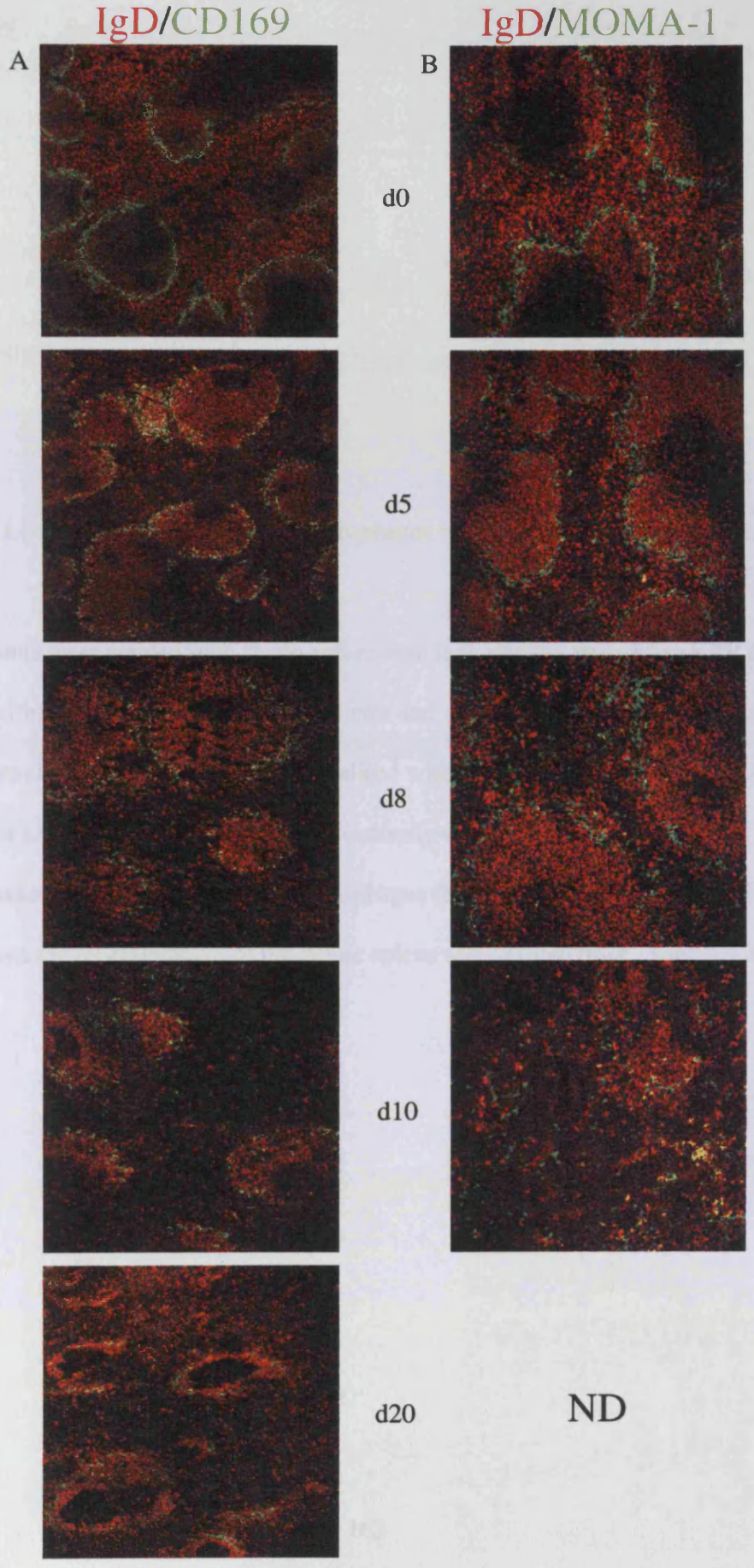
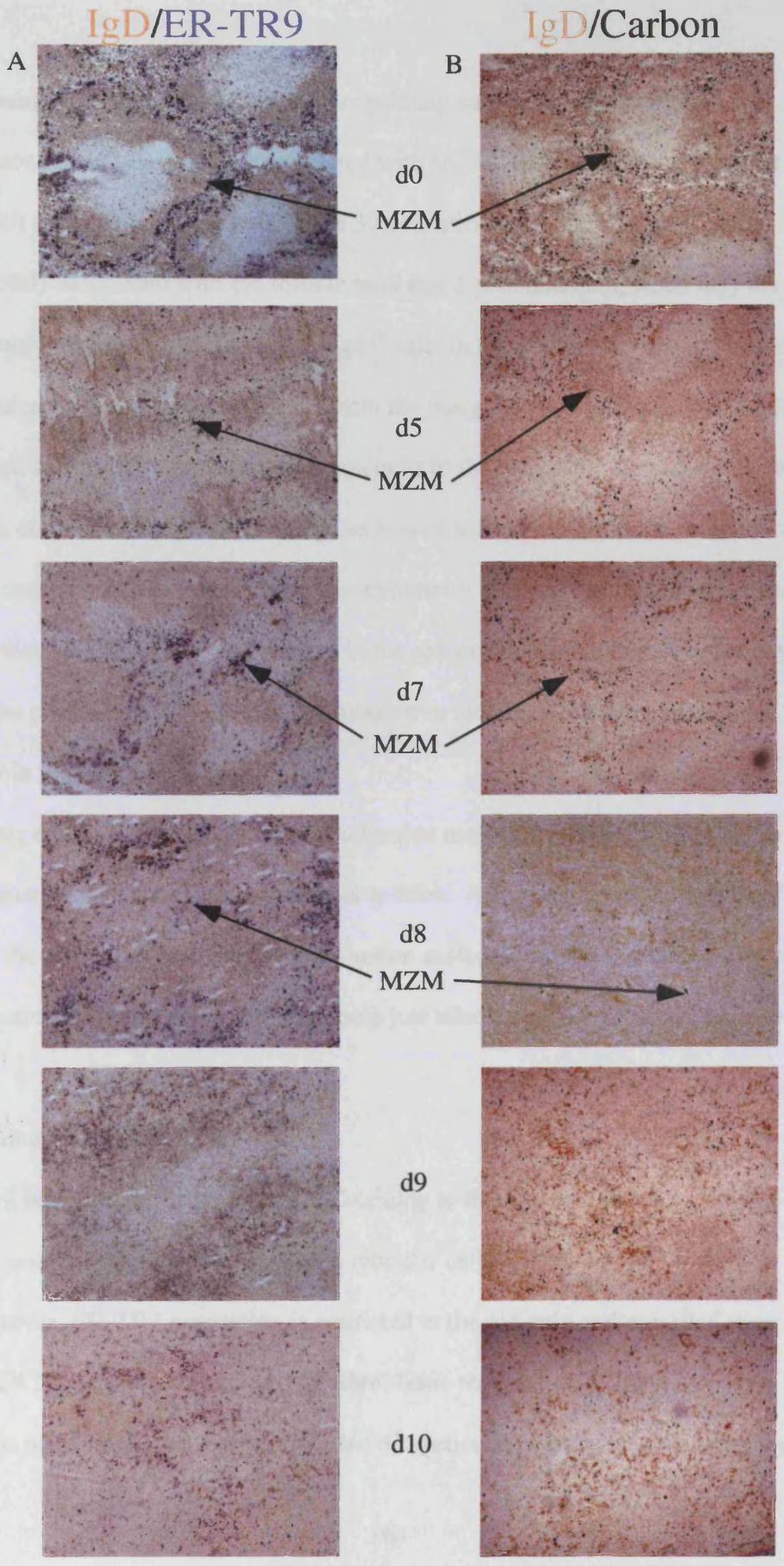

IgD/CD169
IgD/MOMA-1
A
B
d0
d5
d8
d10
d20
ND

Figure 29. Location of marginal zone macrophages in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD, donkey anti-sheep HRP & visualised with DAB (brown) for B cell follicles and rat anti-mouse ER-TR9, rabbit anti-rat biotin, streptavidin ABComplex AP & visualised with Fast Blue (blue) for marginal zone macrophages (A). Mice were injected intravenously with 5% Indian Ink in saline, which is selectively taken up by marginal zone macrophages (black), one day before infection (B). Images shown are representative of the whole spleen and of three mice. Magnification x 30.4


IgD/ER-TR9
IgD/Carbon
A
B
d0
MZM
d5
MZM
d7
MZM
d8
MZM
d9
d10

from the marginal zone, rather than downregulating cell surface molecules.

Marginal zone B cells are closely associated with MZM, and can be clearly seen at the edge of the B cell follicle in naïve mice (Figure 30), identified as IgM^{high}, $IgD^{low/-}$ cells. They remain closely associated with the follicle until day 5 post infection, when they are no longer found in the marginal. Although IgM^{+} cells in the marginal zone may be identified as marginal zone B cells, once removed from the marginal zone location, MZ B cells may be confused with IgM^{+} plasmablasts/plasma cells in the red pulp. This confirms previous work from our laboratory demonstrating the loss of MZ B cells from the marginal zone during acute malaria infection [346]. Flow cytometry analysis has previously revealed, however, that MZ B cells are still present in the spleen, increasing in number at days 5 and 6 before the peak of parasitaemia and decreasing in number at, and just after, peak parasitaemia [346].

Sinus-lining endothelial cells express the adhesion molecule MAdCAM-1 [186], which clearly demarcates the marginal zone in naïve mice. Again beginning around day 5-6 post-infection, the expression pattern of this adhesion molecule changes, until it can be seen as a network pattern encompassing the white pulp just after the peak of infection (Figure 31) [345].

Reticular and endothelial cells

Other work has shown similar patterns of staining to that we see with MAdCAM-1 during infection, over the white pulp area, with a reticular cell marker, ER-TR7 [421]. In our hands however, ER-TR7 expression is restricted to the red pulp and marginal zone (Figure 32). ER-TR7 is an antigen produced by fibroblastic reticular cells that may have a role in chemotaxis of lymphocytes, by forming part of a reticular meshwork making up the

Figure 30. Location of marginal zone B cells and IgM^+ plasma cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and goat anti-mouse IgM FITC (green) for marginal zone B cells/IgM^+ plasma cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4

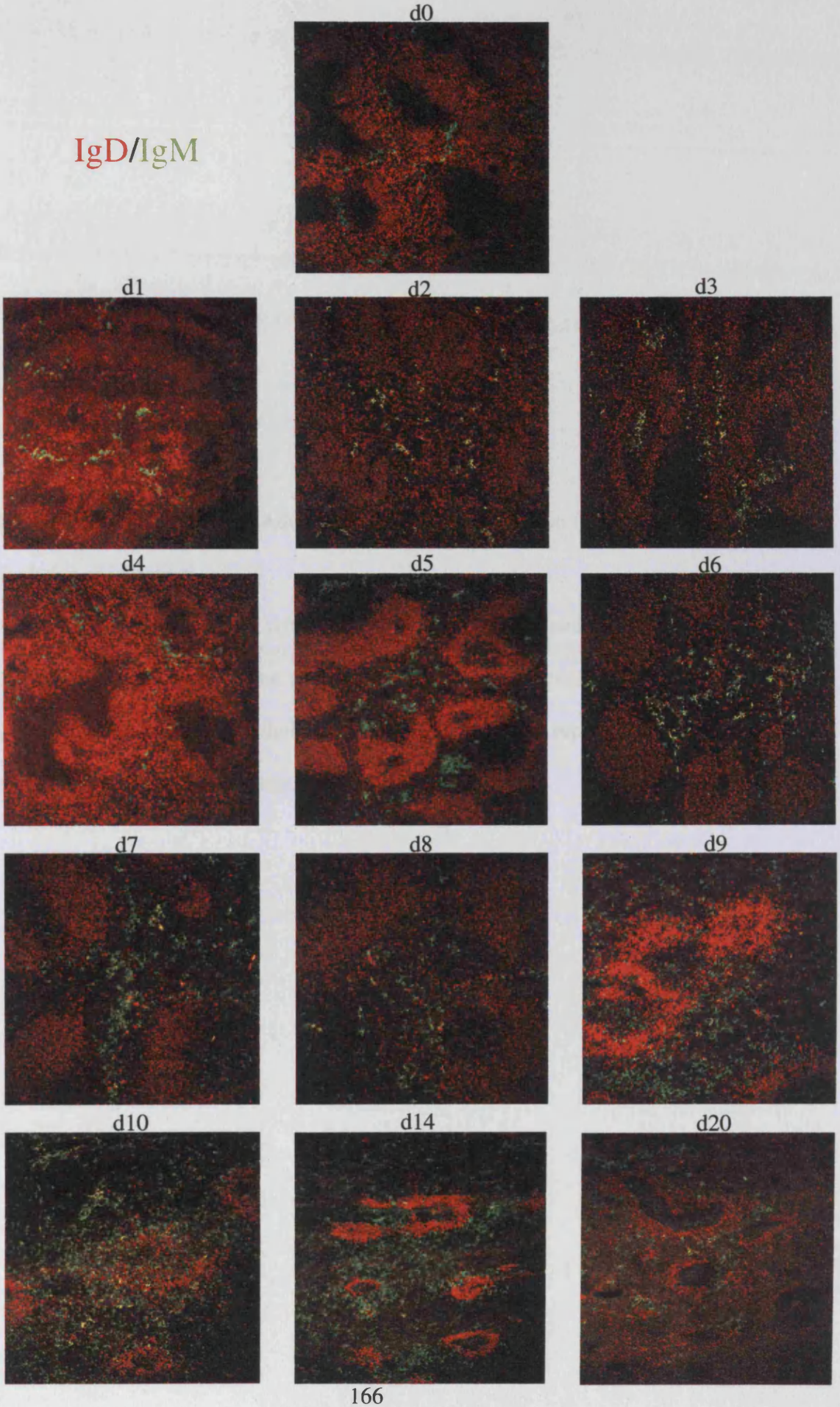

IgD/IgM
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Figure 31. Expression of MAdCAM-1 on endothelial cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse MAdCAM-1 & goat anti-rat IgG Alexa 488 (green) for sinus-lining endothelial cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4

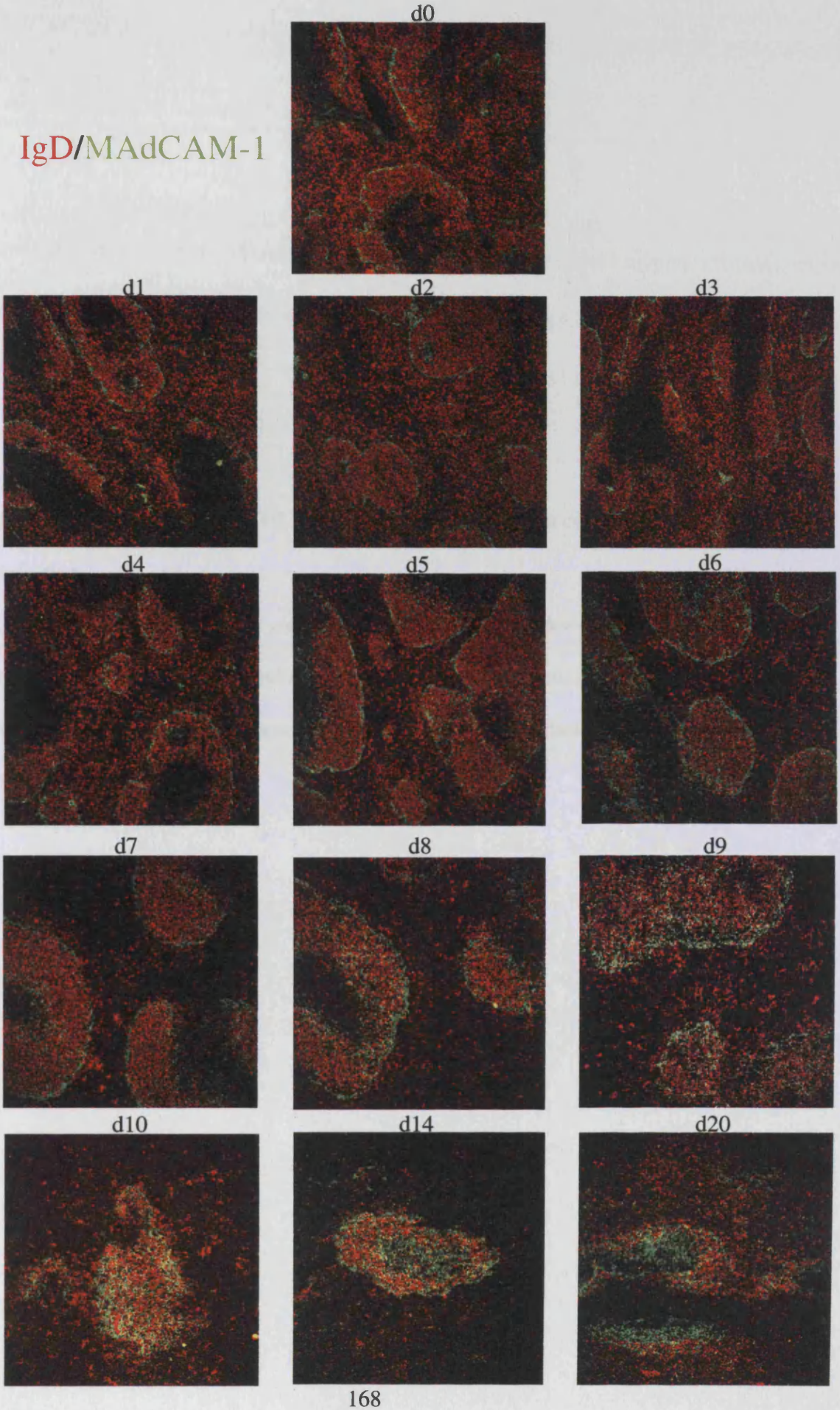
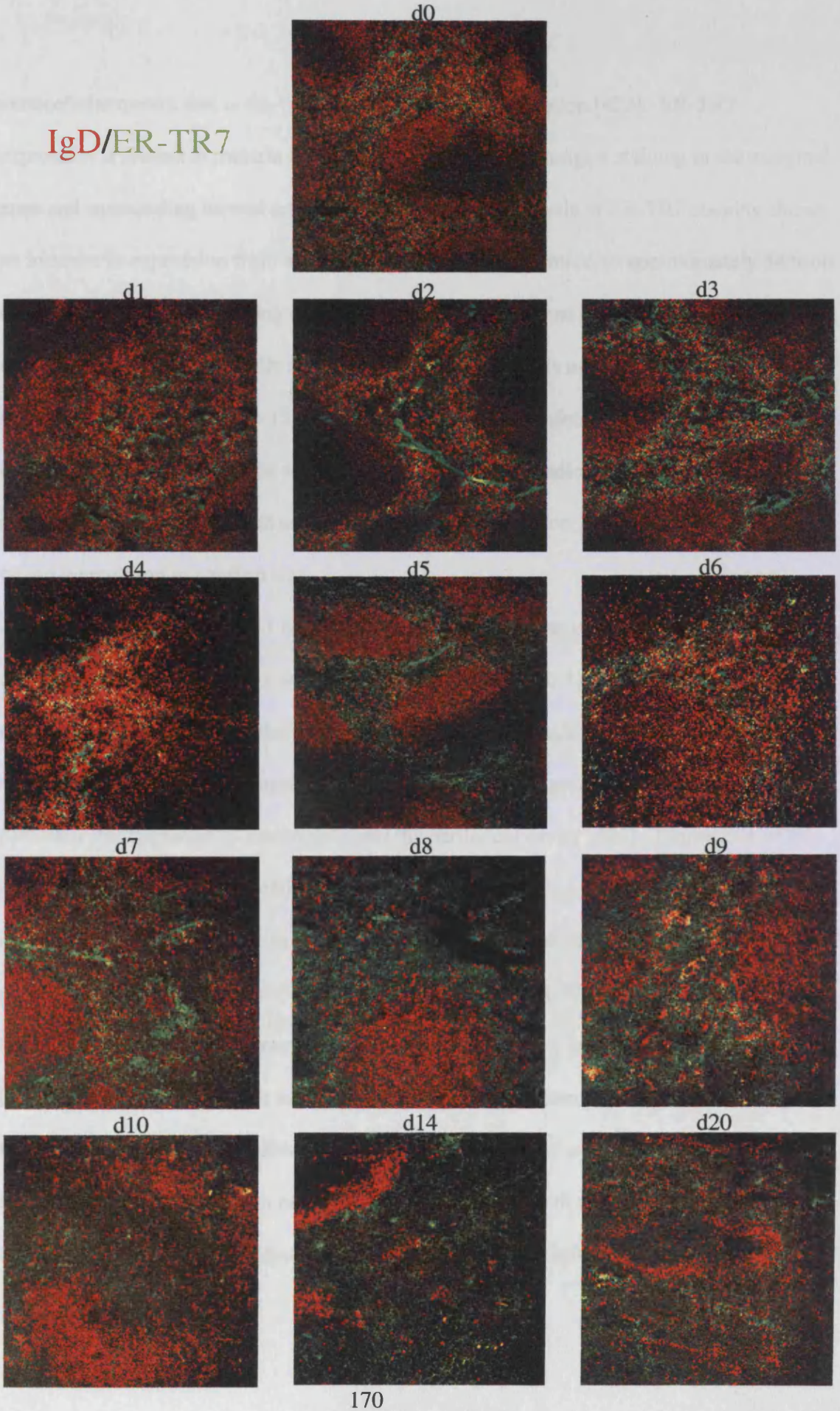

IgD/MAdCAM-1
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Figure 32. Location of ER-TR7^{+} reticular cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse ER-TR7 & goat anti-rat IgG Alexa 488 (green) for reticular cells. Images shown are representative of the whole spleen and of three mice. Magnification x 61

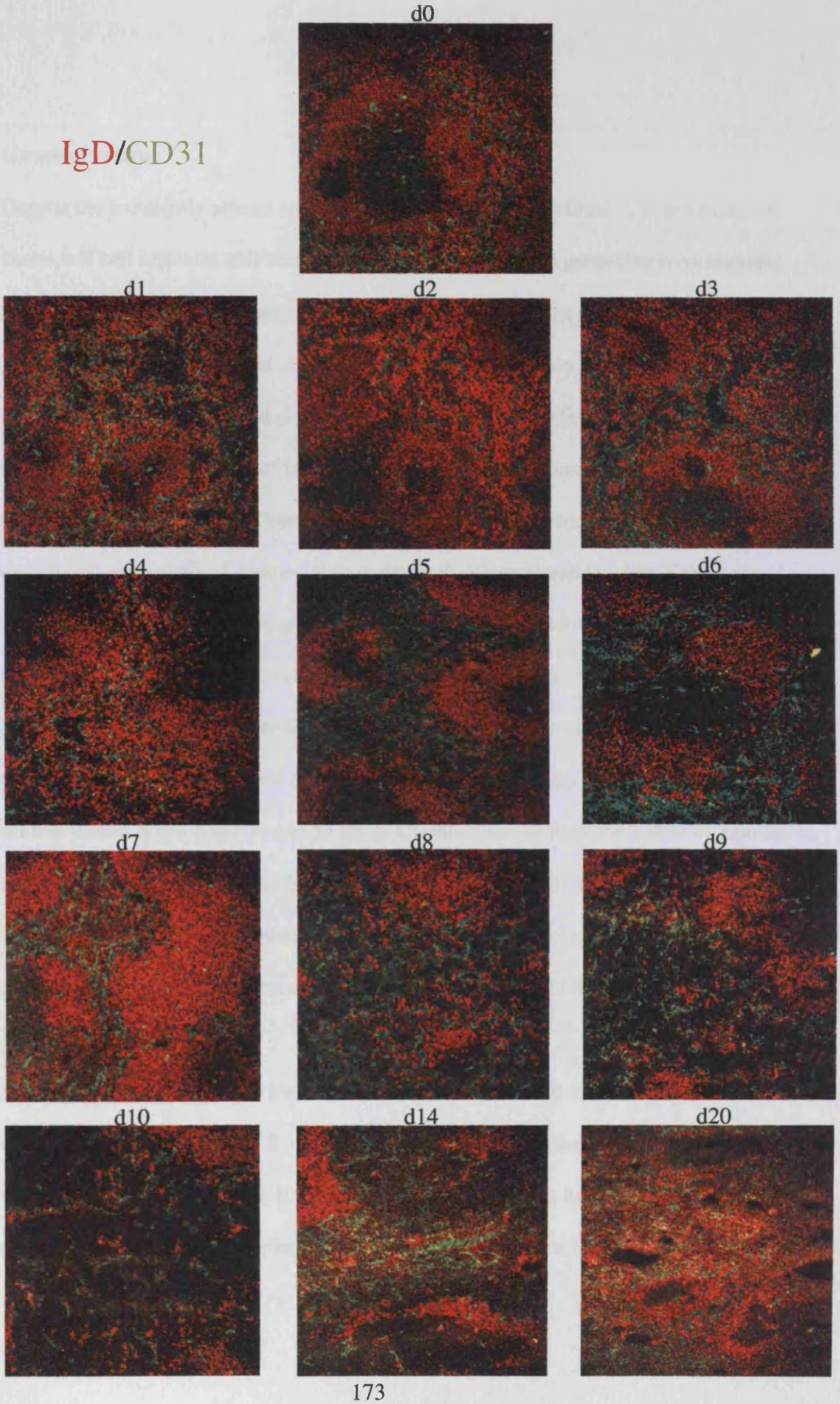

IgD/ER-TR7
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

extracellular matrix that is the "scaffolding" for organ formation [422]. ER-TR7 expression is altered in malaria infected spleens, with the strongest staining in the marginal zone and surrounding central arterioles. Colour picker analysis of ER-TR7 staining shows an increase in expression from approximately 34% in naïve mice, to approximately 54% on day 8 post-infection, indicating either an increase in number of reticular cells or induction of ER-TR7 expression on cells not previously expressing this molecule. Although the spleen increases in size up to 15-fold during acute malaria infection [184], colour picker analysis was conducted on the same area of splenic tissue, indicating an increase in density of ER-TR7 expression as well as in total amount of expression. Further work, however, would be required to confirm this.

As the increased MAdCAM-1 expression does not co-localise with ER-TR7$^+$ reticular cells, other endothelial cell markers were studied. CD31 (PECAM-1) is another adhesion molecule expressed on endothelial cells in the spleen, although not those lining the marginal sinus [423]. It is important for transendothelial migration of lymphocytes, in particular the migration of neutrophils into the peritoneal cavity [424]. Expression of this molecule does not alter in location during the malaria infection. It is found in the red pulp, bridging channels and a little in the T cell zone in naïve mice, and remains mostly in the red pulp throughout the acute infection (Figure 33). As with ER-TR7 expression, the degree of CD31 expression increases from approximately 25% in naïve mice, to approximately 60% by day 8 of infection over the same area of the spleen, indicating an increase in density of CD31 expression, and a possible increase in either number of endothelial cells, or induction of expression of this adhesion molecule, although further work would be required to detail changes in endothelial cell populations during acute malaria infection.

Figure 33. Location of $CD31^+$ endothelial cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD31 & goat anti-rat IgG Alexa 488 (green) for endothelial cells. Images shown are representative of the whole spleen and of three mice. Magnification x 61


IgD/CD31
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Germinal centres

Despite the transiently altered splenic structure and lack of defined T, B and marginal zones, a B cell response still occurs. Mice were not kept in a germ-free environment, therefore some germinal centres (1-2 per B cell follicle) can be seen in uninfected mice. These increase in number and size during the malaria infection, reaching their maximum after the peak of parasitaemia (Figure 34) [346]. There are a few T cells within germinal centres in the first few days of infection (0-6), with a larger number of T cells being visible in larger germinal centres at later time points (day 10 onwards). T cells are always closely associated with germinal centres, even at the peak of parasitaemia when T cells are distributed throughout the red and white pulp (Figure 34). Between days 7 and 9 post-infection, encompassing the period of peak parasitaemia, many germinal centre cells are present in the spleen, however they are mostly distributed throughout the red pulp rather than forming discrete germinal centres within follicles. By day 10 post-infection, germinal centres within B cell follicles can be seen. Germinal centre formation after immunisation or viral infection can normally be seen by day 6-10 [151, 210], although they can also be seen as early as day 4 [151], therefore the scarcity of germinal centres between days 7 and 9 post-infection points towards a slight delay in germinal centre formation.

Plasma cells

Small foci of extra-follicular plasma cells are present in naïve mice and in the first week of infection, however from day 8 onwards, larger numbers of plasma cells can be seen in the red pulp (Figure 35). By day 10 of infection the plasma cells have spread to the white pulp region as well. The peak of plasma cell numbers in the spleen has been shown to be around

Figure 34. Location of germinal centres, and their location in relation to T cells, in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (blue) for B cell follicles, rat anti-mouse CD3 & chicken anti-rat Alexa 647 (red) for T cells and peanut agglutinin biotin & streptavidin FITC (green) for germinal centres. Images shown are representative of the whole spleen and of three mice. Areas of red pulp (RP) and white pulp (WP) are indicated. Magnification x 30.4

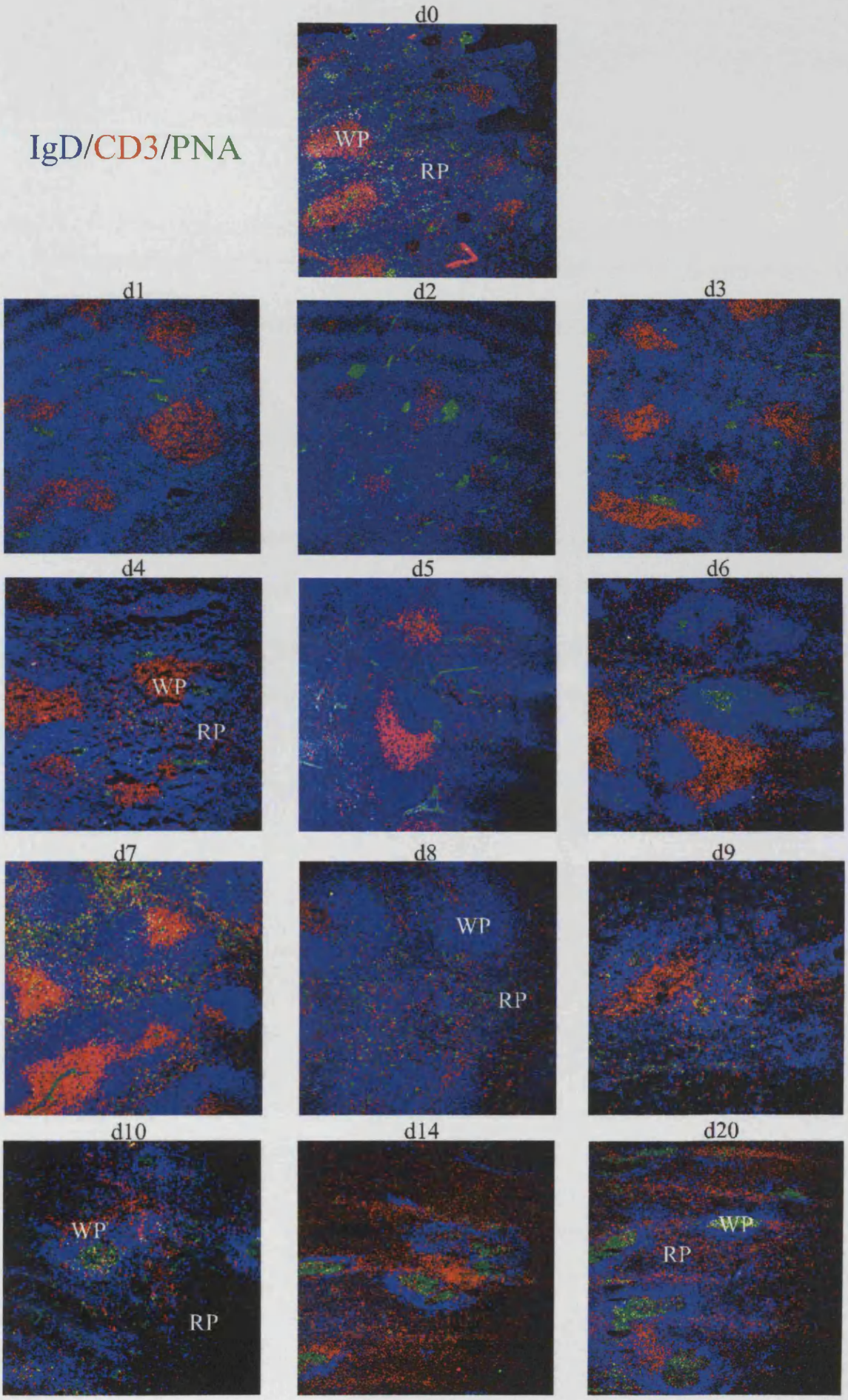
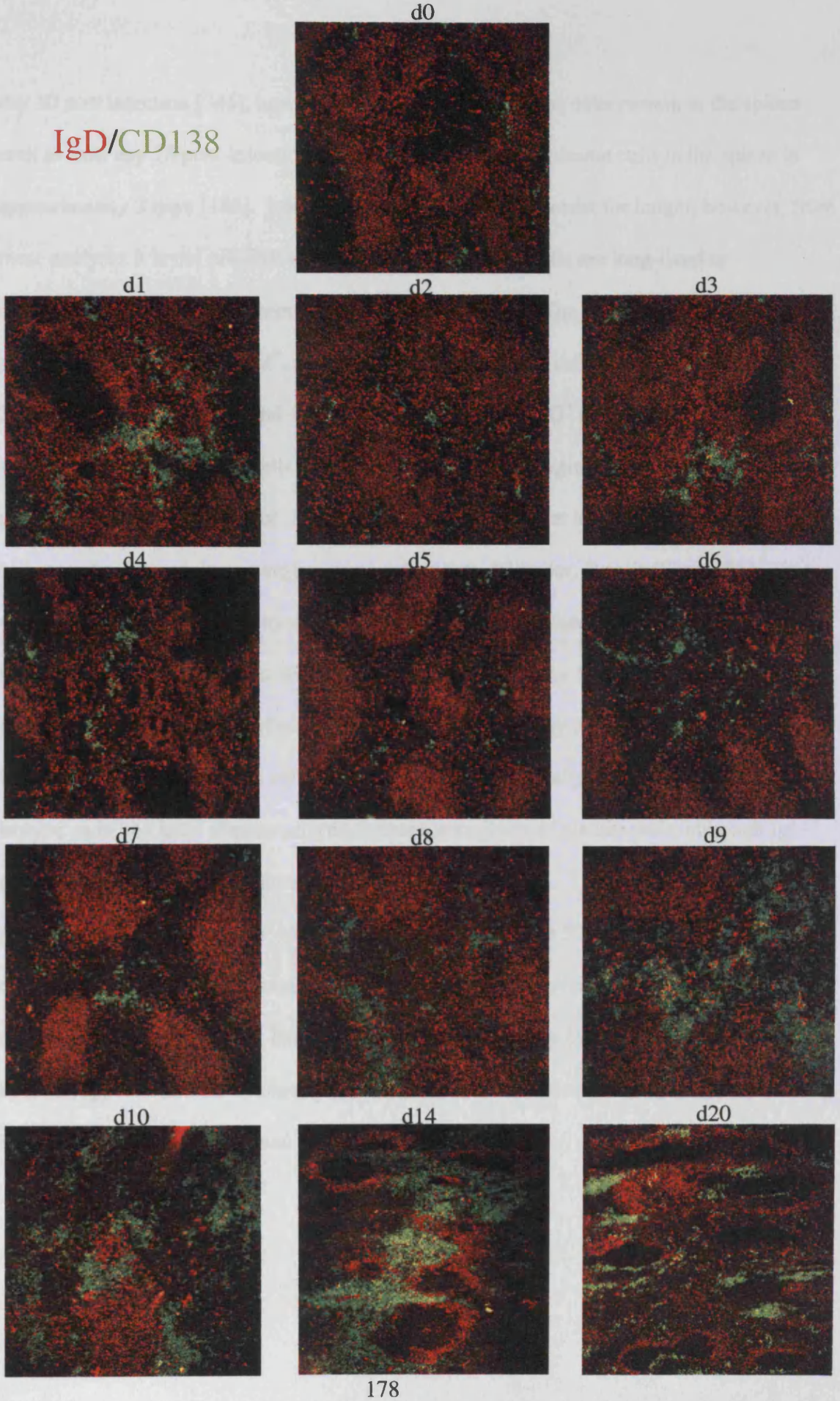

IgD/CD3/PNA
d0
WP
RP
d1
d2
d3
d4
WP
RP
d5
d6
d7
d8
WP
RP
d9
d10
WP
RP
d14
d20
WP
RP

Figure 35. Location of plasma cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD138 & goat anti-rat IgG Alexa 488 (green) for plasma cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4


IgD/CD138
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

day 10 post infection [346], however large numbers of plasma cells remain in the spleen until at least day 20 post-infection. The average lifespan of plasma cells in the spleen is approximately 3 days [148]. Long-lived plasma cells may persist for longer, however, from these analyses it is not possible to tell whether the plasma cells are long-lived or continuously being formed throughout the acute infection. The majority of plasma cells present in naïve mice are IgM^+, although a few IgG^+ plasma cells are also present (Figures 36 & 37). Between days 1 and 4 post-infection, very few IgG^+ plasma cells are present, and the majority of plasma cells are IgM^+. IgM^+CD138^- marginal zone B cells are located close to extra-follicular foci of plasma cells, suggesting that at least some of these plasma cells may be derived from marginal zone, rather than follicular, B cells (Figure 36). Up to day 7 of infection, the majority of plasma cells are non-switched, IgM^+ cells, although IgG^+ plasma cells can be seen in increasing numbers as early as day 5 post-infection. Colour picker analysis shows 38% of plasma cells being IgM^+ on day 7 post-infection, compared to only 9% IgG^+. As plasma cell numbers increase dramatically, from day 8 post-infection, isotype switched IgG^+ plasma cells constitute the majority of plasma cells, although IgM^+ plasma cells are still visible through to day 20 post-infection. The peak number of IgG^+ plasma cells appears to occur on day 14 post-infection, when 50% of plasma cells are IgG^+ compared to 23% IgM^+, although this is a rough analysis of pixel numbers rather than cell numbers, using only a single image, and should be backed up by more extensive analysis by histology and/or flow cytometry for a full characterisation of antibody isotype switching in the spleen during *P.chabaudi* infection.

Figure 36. Location of marginal zone B cells and IgM^+ plasma cells in the spleen during acute *P.chabaudi* infection

Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (blue) for B cell follicles, rat anti-mouse CD138 & chicken anti-rat Alexa 647 (red) for plasma cells and goat anti-mouse IgM FITC (green) for IgM^+ plasma cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4

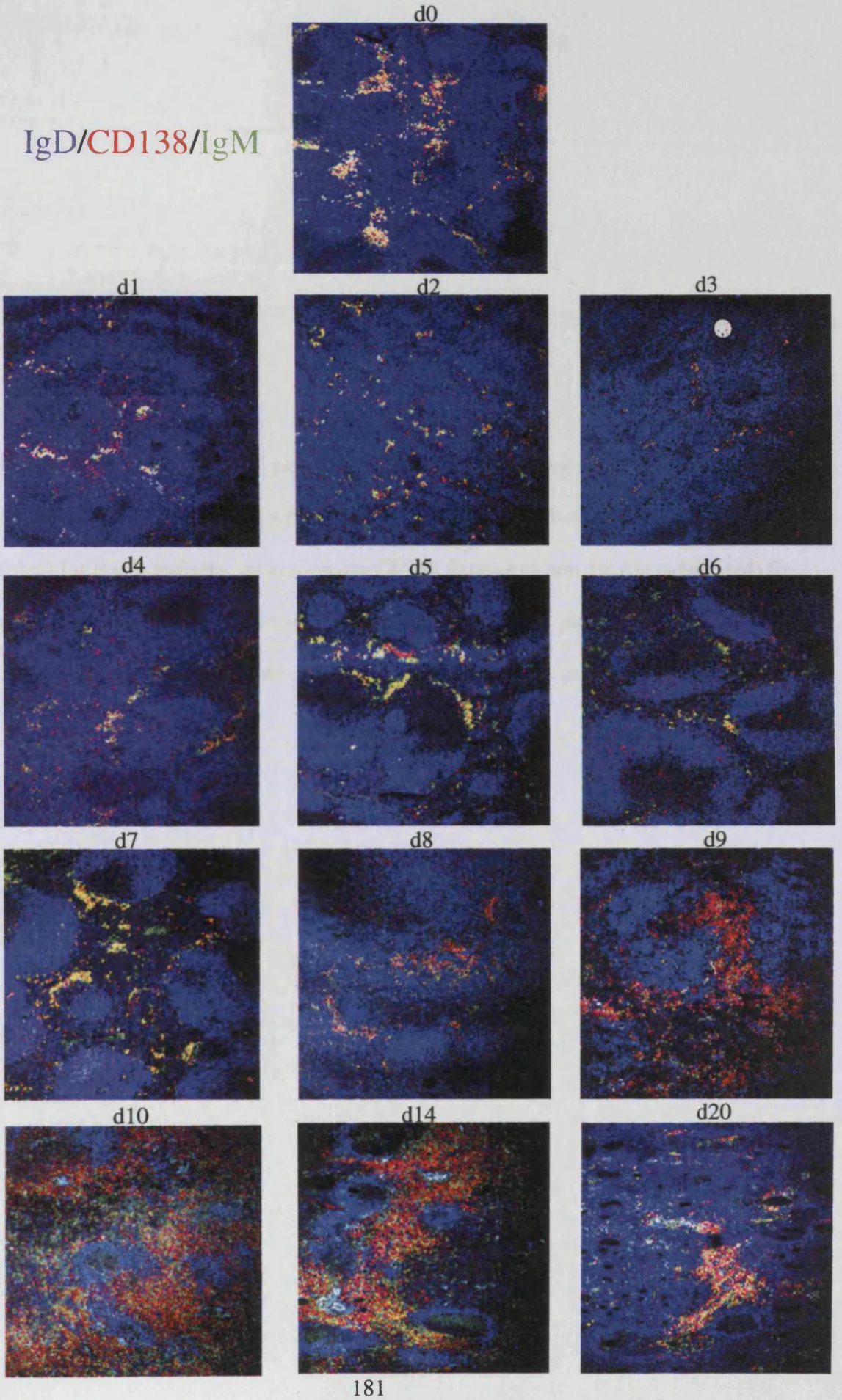
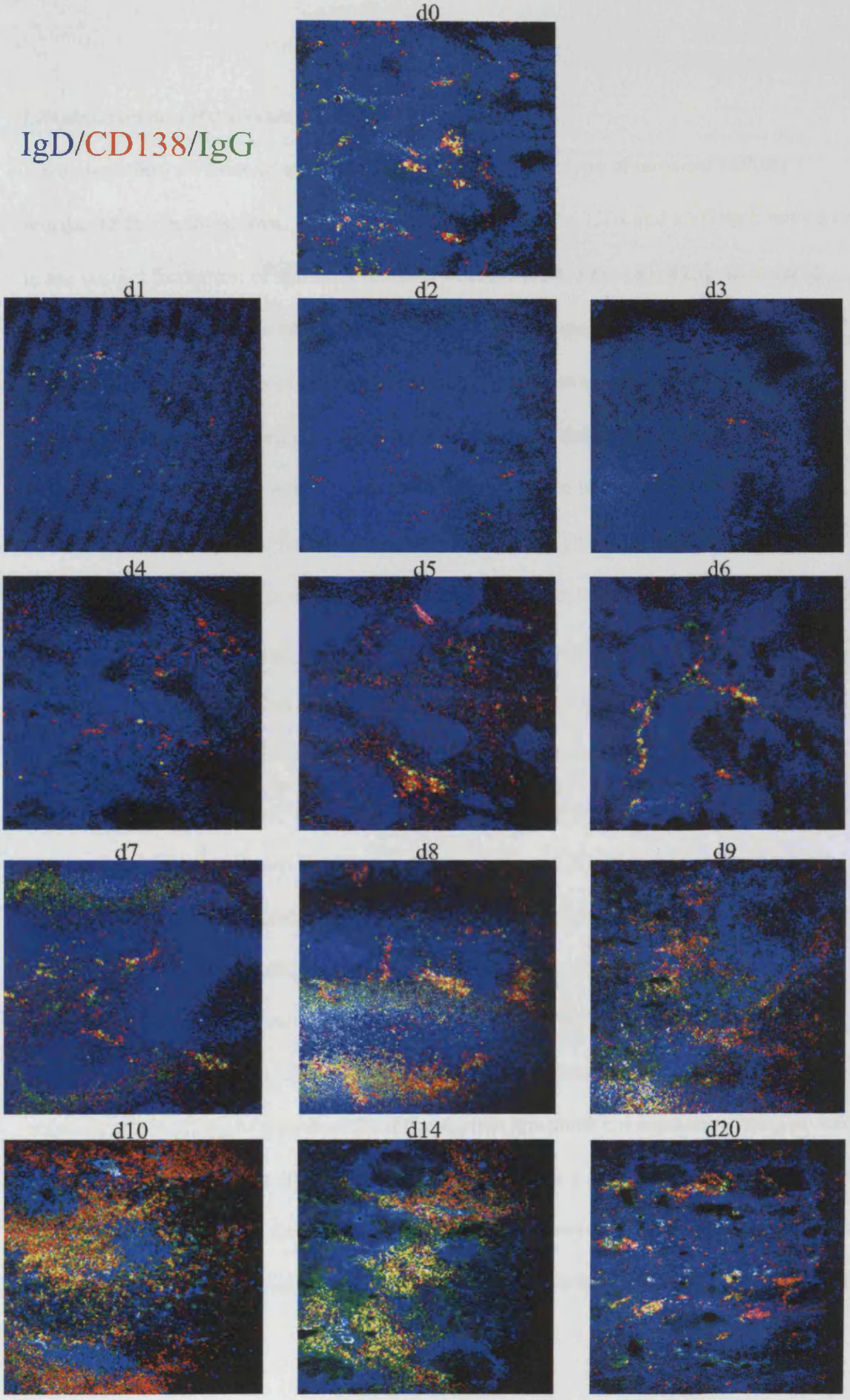

IgD/CD138/IgM
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Figure 37. Location of IgG^+ plasma cells in the spleen during acute *P.chabaudi* infection Spleen sections were stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 568 (blue) for B cell follicles, rat anti-mouse CD138 & chicken anti-rat Alexa 647 (red) for plasma cells and goat anti-mouse IgG FITC (green) for IgG^+ plasma cells. Images shown are representative of the whole spleen and of three mice. Magnification x 30.4


IgD/CD138/IgG
d0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
d14
d20

Lymphotoxin and the splenic microarchitecture

Taken together, all these changes provide a unique phenotype of transient cellular reorganisation in the spleen. It has been shown that TNFα, LTα and LTβ each have a role in the correct formation of splenic microarchitecture [164, 171, 181, 425]. In order to investigate the importance of splenic changes in primary malaria infection, we attempted to prevent the reorganisation of splenic structure by administration of an agonistic LTβR antibody. Changes in splenic microarchitecture become detectable between days 5 and 6 post-infection, during the exponential growth phase of the blood-stage infection. Day 4 post-infection was chosen for the administration of the LTβR agonist antibody as being prior to the changes in splenic structure becoming visible, but close enough to this critical time period to be able to have an effect. Unfortunately, administration of this antibody did not have any effect on either splenic structure or parasitaemia in the primary infection (Figure 38). T and B cell zones still merge and there is no visible difference in splenic microarchitecture between mice treated with anti-lymphotoxin β agonist antibody (Group C), control antibody (Group B) or untreated mice (Group A). It was hypothesised that retention of the correct splenic microarchitecture during primary infection might improve immunity to secondary infection, and that if splenic microarchitecture could not be preserved during the primary infection, a more subtle effect of the lymphotoxin agonist antibody might have a visible effect on immunity and splenic microarchitecture during secondary infection. Administration of the agonist lymphotoxin antibody during primary infection had no effect on splenic microarchitecture during a secondary infection (Figure 39), however mice given the lymphotoxin agonist antibody had lower parasitaemia during the secondary infection (Figure 39A). Although this difference is slight, it suggests that

Figure 38. Effect of lymphotoxin agonist on primary *P.chabaudi* infection.

Spleen sections were taken at day 10 post-infection and stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD3 & goat anti-rat IgG Alexa 488 (green) for T cells. Images shown are representative of the whole spleen. Magnification x 30.4

A) Primary infection parasitaemia in untreated (—), control antibody treated (—) and lymphotoxin agonist antibody treated (—) mice

Red arrow indicates time of agonist lymphotoxin antibody administration

B) Splenic microarchitecture in wild-type mice

C) Splenic microarchitecture in control antibody treated mice

D) Splenic microarchitecture in lymphotoxin agonist antibody treated mice

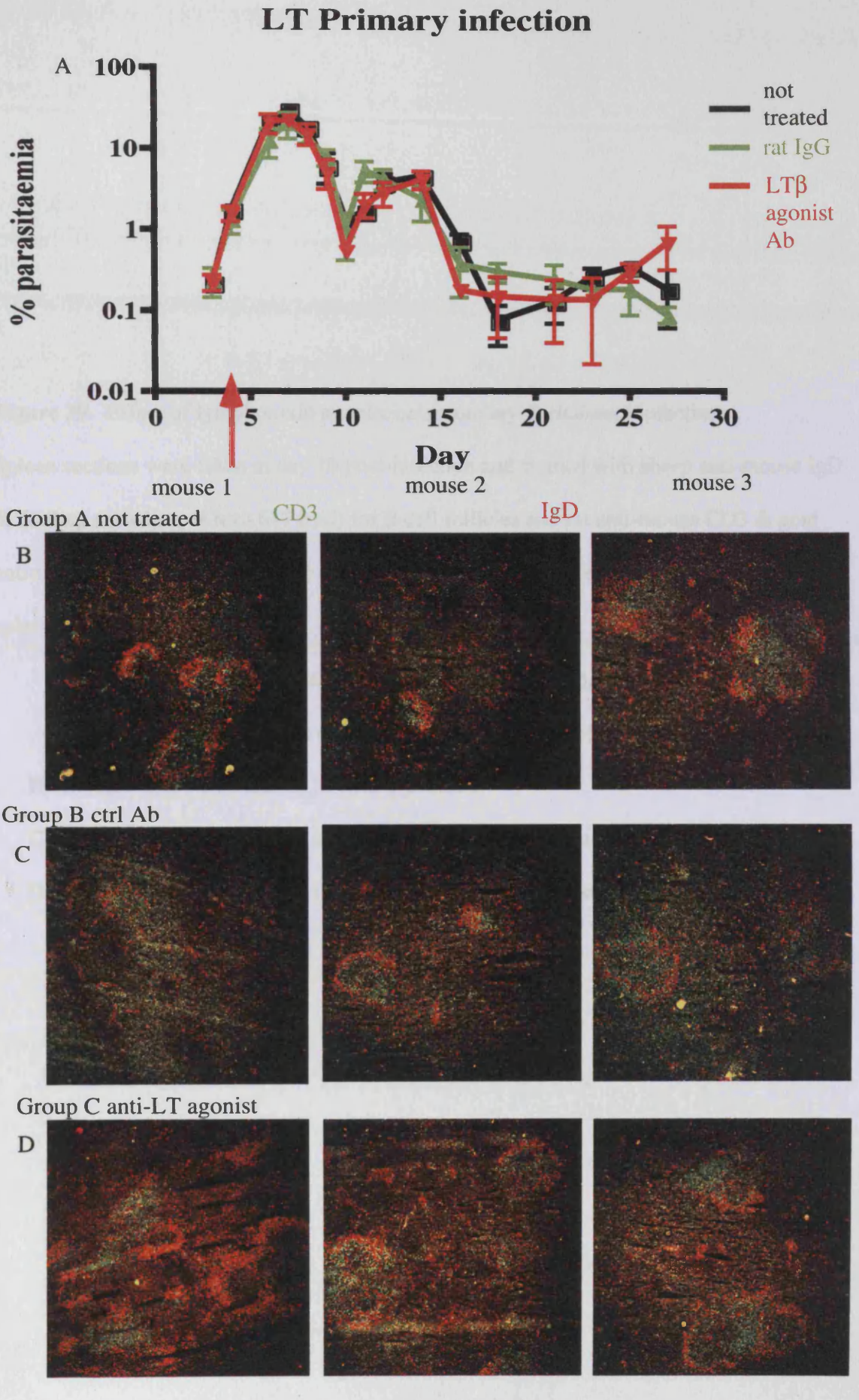

LT Primary infection
A
100
10
1
0.1
0.01
% parasitaemia
0
5
10
15
20
25
30
Day
not treated
rat IgG
LTβ agonist Ab
mouse 1
mouse 2
mouse 3
Group A not treated
CD3
IgD
B
Group B ctrl Ab
C
Group C anti-LT agonist
D

Figure 39. Effect of lymphotoxin agonist on secondary *P.chabaudi* infection.

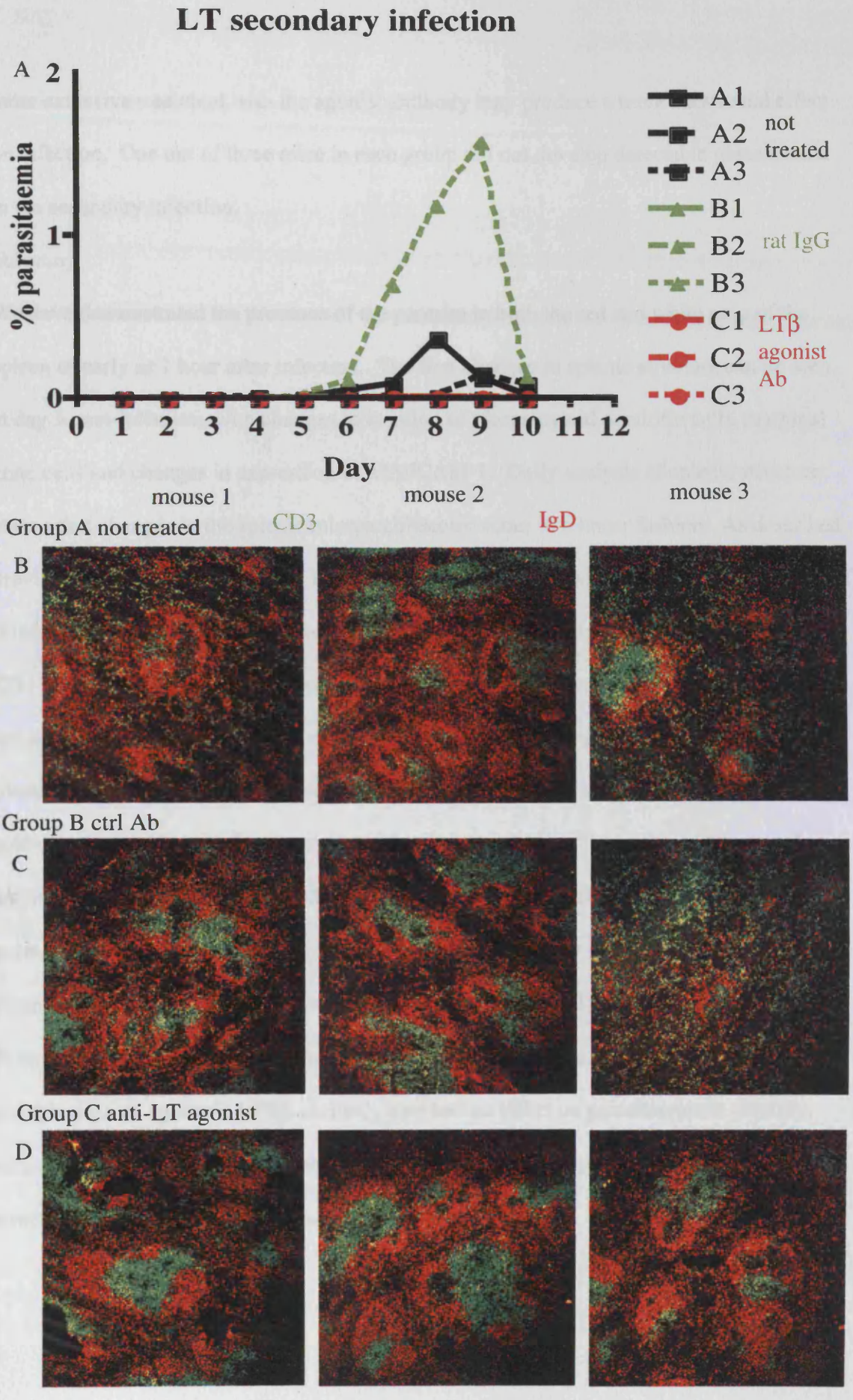
Spleen sections were taken at day 10 post-infection and stained with sheep anti-mouse IgD & donkey anti-sheep Alexa 647 (red) for B cell follicles and rat anti-mouse CD3 & goat anti-rat IgG Alexa 488 (green) for T cells. Images shown are representative of the whole spleen. Magnification x 30.4

A) Secondary infection parasitaemia in untreated (▬), control antibody treated (▬) and lymphotoxin agonist antibody treated (▬) mice

B) Splenic microarchitecture in wild-type mice

C) Splenic microarchitecture in control antibody treated mice

D) Splenic microarchitecture in lymphotoxin agonist antibody treated mice


LT secondary infection
A
% parasitaemia
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Day
A1
A2
A3
not treated
B1
B2
B3
rat IgG
C1
C2
C3
LTβ agonist Ab
mouse 1
mouse 2
mouse 3
Group A not treated
CD3
IgD
B
Group B ctrl Ab
C
Group C anti-LT agonist
D

more extensive treatment with the agonist antibody may produce a more substantial effect on infection. One out of three mice in each group did not develop detectable parasitaemia in the secondary infection.

Summary

We have demonstrated the presence of the parasite in both the red and white pulp of the spleen as early as 1 hour after infection. The first changes in splenic structure can be seen at day 5 post-infection, with changes in location of plasmacytoid dendritic cells, marginal zone cells and changes in expression of MAdCAM-1. Daily analysis of splenic structure shows that changes in the splenic microarchitecture occur in a linear fashion. As described previously [345, 346], alterations in splenic structure are maximal between days 8 and 10 of infection, around the peak of parasitaemia, when T and B cell zones are merged, $CD8^-$ $CD11c^+$ numbers peak, large germinal centres and numerous extra-follicular plasma cells are seen. Isotype switching from a majority of IgM^+ plasma cells to a majority of IgG^+ plasma cells occurs on day 8 post-infection. As the parasite is cleared, changes in the splenic microarchitecture begin to reverse by day 14 post-infection, with cellular number and location becoming similar to that in naïve mice, although the spleen remains permanently enlarged. These changes were hypothesised to be due to a temporary loss of signalling through the LTβ pathway, however expression of LTβ, LTβR or CXCR5 (Figure 7) could not be detected by histology in either naïve or infected spleens. Administration of a single dose of agonistic LTβR antibody also had no effect on parasitaemia in primary infection, little effect on parasitaemia in secondary infection, and no effect on splenic structure in either primary or secondary infections.

Discussion

During malaria infection the spleen increases up to 15-fold in weight [184], and approximately 7-fold in lymphocyte numbers (Chapter 5, Figure 43, [346, 392]), and becomes much darker in colour due to the deposition of haemozoin (malaria pigment). Although it has previously been shown that striking but transient changes in location of the normal populations of splenic cells occur during acute malaria infection [184, 345, 346, 416], the point at which these changes first become evident had not been established. Similarly, the order in which different cell populations change location was not known. We therefore undertook a detailed analysis of the timing and extent of changes in splenic cell populations during the acute phase of malaria infection. The use of immunofluorescence rather than conventional histology enabled residual B cell follicles at the peak of microarchitecture alterations to be identified more easily (compare Figures 19 & 29).
In naïve mice, dendritic cells are found in the red pulp, marginal zone and T cell zone [60], with $CD8^-$ DCs preferentially found in the red pulp, MZ and bridging channels, and $CD8^+$ DCs in the T cell zone (review [55]). They migrate from the MZ and red pulp to the T cell zone during infection [45, 62] and in response to LPS [45], for antigen presentation. Migration of dendritic cells in the spleen during malaria infection has been demonstrated previously [415], however no one has characterised the migration of $CD8^+$ and $CD8^-$ dendritic cell subsets or plasmacytoid DCs in response to malaria. It is possible that the large number of DCs seen in acute *P.chabaudi* infection have proliferated within the spleen rather than migrated from the blood. The increase in dendritic cell numbers in the spleen could, however, be due to a combination of influx of cells from the blood and development

from dendritic cell precursors within the spleen [61]. The preferential accumulation of $CD8^-CD11c^+$ DC over $CD8^+CD11c^+$ DC has interesting functional implications as the two subsets have been shown to express different cell surface molecules [426], may have different co-stimulatory requirements during antigen encounter [427] and may perform different functions (e.g. $CD8^+$ DC preferentially endocytose apoptotic cells) [63]. They are currently thought to be distinct populations rather than the same population at different stages of development [69] (review [71]).

The ability of these dendritic cell subsets to present malaria antigens has been investigated, and it has been found that $CD8^-$ DC present parasite antigens *in vivo* better than $CD8^+$ DC at the peak of infection [61]. In contrast, $CD8^+$ DC present malaria peptide better than $CD8^-$ DC *in vitro*, therefore it is possible that the location of $CD8^-$ DCs in the marginal zone and red pulp gives them better access to pRBC, enabling them to present malaria antigens better. We have shown that greater numbers of $CD8^+$ DCs undergo apoptosis than $CD8^-$ DC *in vivo*, and $CD8^+$ DCs are known to have a shorter lifespan than $CD8^-$ DCs [64], which may also affect their antigen presenting capacity [61]. $CD8^+$ DCs have been suggested to be less phagocytic than $CD8^-$ DCs (review [55]), although studies showing that $CD8^+$ DCs take up and present antigen better than $CD8^-$ DCs *in vitro* [61] would seem to contradict this. We have shown that $CD8^-$ DCs, not $CD8^+$ DCs, are capable of inducing T cells to produce IL-4 and IL-10, indicating a specific role for $CD8^-$ DCs in initiating the switch towards Th2 responses that occurs between two and three weeks post-infection, after the peak of the acute malaria parasitaemia [61, 103].

Plasmacytoid DCs are important for immune responses to viral infections, as they produce high levels of IFNα [80]. They are not as highly phagocytic as conventional DCs however,

and therefore do not present antigens as well [67]. Recent work has shown that pDCs may be subdivided into two different subsets [68], however for the purposes of this work they have been treated as a single population.

This is the first study of pDCs in the spleen during infection. The location of pDCs in the spleen does not change dramatically over the course of the acute malaria infection; they remain in the red pulp throughout. There is also not the dramatic change in pDC number that is seen with conventional DCs at peak parasitaemia, but instead an increase in pDCs later between days 9 and 20 (Figure 24). pDCs appear to become more closely associated with the marginal zone on days 5 to 7 post infection, at the site of blood (and pRBC) entry into the spleen, which may indicate a role for them in taking up parasites and presenting malaria antigens to lymphocytes. Studies of antigen presentation by pDCs in malaria have, however, shown little presentation of malaria antigens to T cells by pDC, although IFNα is produced by pDCs in response to malaria infection and after *in vitro* culture with pRBC (C.Voisine *et al*, in preparation).

The marker used to identify pDCs, 120G8, can also be expressed on B cells activated by IFNα [80]. Expression levels are however lower on B cells (at least one log MFI) [80]. There is very little co-staining of 120G8 with the naïve B cell marker IgD, nor does the staining pattern resemble that of germinal centre B cells or plasma cells, therefore the $120G8^{+}$ cells shown here are all pDCs.

pDCs may alternatively be activated by the parasite through TLRs, inducing migration into the marginal zone and thereby bringing them closer to IFNα-responsive cells. They do not appear to increase in numbers in the white pulp, however with the migration of B and T cells into the red pulp, interactions may occur in unusual locations. IFNα produced by

pDCs affects B cell responses directly by enhancing isotype switching to IgG [428], can promote B cell activation in the absence of T cells [429] and, together with IL-6, induces plasma cell differentiation [55]. IFNα can also promote expression of the B cell activating molecules BAFF and APRIL on conventional dendritic cells, further enhancing antibody isotype switching [428]. It would be interesting to deplete pDCs to determine the extent of their role in the B cell and plasma cell response to malaria infection.

We have observed a loss of marginal zone B cells, MZM and MMM from the marginal zone during acute malaria infection. Although some MZM and MMM remain in the marginal zone, the majority are lost from this location and their fate is unknown. Previous work has shown that although MZ B cells are lost from the marginal zone during acute malaria infection, they remain in the spleen and do not undergo apoptosis [346]. IgM^{high} cells are found close to plasma cells, indicating that MZ B cells may be responsible for the large numbers of extra-follicular plasma cells seen in the spleen around the peak of infection.

MZM and MMM may likewise remain in the spleen, spread throughout the red pulp and therefore difficult to distinguish by histology. Alternatively, they may phagocytose pRBC directly, or phagocytose other cells that are undergoing apoptosis after taking up pRBC (e.g. the large proportion of $CD8^+$ DCs that undergo apoptosis within 5 days post-infection, when MZM and MMM begin to be lost from the marginal zone). With the availability of fluorescently labelled *P.chabaudi*, it should be possible to determine whether MZM and MMM directly phagocytose parasites, or cells that have phagocytosed parasites.

Phagocytosis of apoptotic cells has been shown to promote the survival of macrophages and inhibit proliferation [430]. Phagocytosis can also trigger the migration of tissue-

resident macrophages to the draining lymph node in order to present antigen [431], however this does not explain the migration of macrophages resident in the spleen away from a site ideally suited to antigen presentation. Evidence from other systems [172, 174, 179] indicates that loss of macrophages from the marginal zone is likely to be due to excess TNFα causing downregulation of CCL21 expression.

Marginal zone cells are thought to be involved in the early response to blood borne antigens and T-cell independent type-2 antigens [432]. Marginal zone B cells, which are lost from the marginal zone on macrophage depletion, induce T cell responses better than follicular B cells after immunisation with protein antigens [204]. They are also more numerous than follicular B cells, 2-3 fold more in the rat [242]. MZ B cells are also thought to be largely responsible for the initial burst of IgM production [130], with germinal centre derived plasma cells of both MZ and follicular B cell origin being responsible for the later switch to IgG isotypes [133]. MZ B cells have been implicated in the transport of antigen into B cell follicles and the establishment of germinal centres [133, 203]. MZ B cells are capable of producing IgG isotypes, which are reduced in the response to TI-II antigens in mice lacking MZ B cells [131].

MZ B cells are in close contact with MZM in the outer ring of the marginal zone, and it has been suggested that they are dependent on MZM and follicular B cells for their location in the marginal zone [242, 414, 433]. Lack of MZM can also affect the location of follicular B cells entering the spleen [434]. However, MZ B cells reappear in the spleen 12-16 days after macrophage depletion and about 2 weeks before MZM [435], and are not affected by elimination of MZM [436], indicating that MZ B cells are not dependent on MZM for their location in the marginal zone.

MZM are thought to be responsive to the chemokine CXCL13, but to be retained in the marginal zone by preferential responsiveness to sphingosine 1-phosphate receptor 1 ($S1P_1$), a receptor that binds phospholipids present in the blood. Whilst $S1P_1$ signalling occurs, MZM remain in the marginal zone, close to the source of this signal in the blood in the marginal sinus. If $S1P_1$ signalling is blocked, MZM can then respond to CXCL13 and migrate into the B cell follicle [437]. Steiniger and Barth [183] speculate that sialoadhesin-positive macrophages in human spleens can leave their position surrounding the sheathed capillaries and migrate into the perifollicular region under certain conditions. If CXCL13 signalling is blocked, MZM may migrate into the blood in response to $S1P_1$ signalling, and return to the marginal zone only when CXCL13 signalling is sufficiently restored. We hypothesise that signalling through the LTβ pathway is disrupted during acute malaria infection, which would lead to a loss of CXCL13 signalling (Figure 7), and could therefore result in the loss of MZM into the blood stream in response to $S1P_1$ signalling. If these cells do migrate from the spleen, there are no clues to their destination. MZM can be visualised by uptake of fluorescently labelled Ficoll particles [438], and could perhaps be tracked during migration this way.

The significance of losing macrophages and MZ B cells from the marginal zone during acute *P.chabaudi* infection is unclear, however macrophages of the marginal zone could reasonably be expected to have a role in removal of parasites. Maintaining macrophages in the marginal zone during acute infection could potentially increase the removal of parasites from the blood. One obvious experiment to investigate the role of these cells during malaria infection would be to selectively deplete these cell populations, and observe the effect of this depletion on infection.

It is not possible to deplete a single population of macrophages, however after depletion with clodronate liposomes, splenic macrophages repopulate the MZ with different kinetics for each subset [435]. Red pulp macrophages reappear one week after depletion, MMM and MZ B cells after two weeks. MZM reappear 1 month after depletion, but do not have full functional capacity [435].

Macrophages of the marginal zone have been shown to have a role in the immune response to *Listeria monocytogenes*, but are not essential for this response [411, 413]. Lack of macrophages of the marginal zone reduces clearance of viral infections [210, 412]. Similarly, depletion of all macrophages by intravenous injection of silica increases susceptibility of C57BL/6 mice to *Plasmodium chabaudi* (AS) infection [410]. The response to a bacterial T-cell dependent antigen also drops sharply after macrophage depletion but returns to control levels on repopulation of MMM [439].

In *Leishmania donovani* infection, MZM are lost from the marginal zone but MMM remain [172]. There are also changes in lymphocyte trafficking into the spleen during *L.donovani* infection which have been at least partially attributed to the loss of MZM [172], and other authors have also suggested a role for MZM and MMM in lymphocyte trafficking into the white pulp [414, 434]. We have not studied lymphocyte migration into the spleen in malaria infection, however the extent of changes in splenic microarchitecture and the closure of splenic circulation [184, 306, 416] would suggest that lymphocyte migration is almost certainly altered during acute infection. It would, however, be difficult to determine the contribution of MZM and MMM to such an effect.

Some changes in splenic microarchitecture can be artificially induced by administration of LPS [45, 440]. LPS administration causes transient loss of MZM and movement of MMM

into B cell follicles, reversing within 48 hrs [349]. The receptor for LPS is Toll-like receptor 4 (TLR4), which is thought to associate with SIGNR1 (identified with MAb ER-TR9) on MZM [62]. This could induce the migration of MZM into follicles in response to bacterial infection as an alternative mechanism for transporting antigen into the white pulp. On a similar note, haemozoin has been shown to activate DCs through TLR9 [350]. Splenomegaly is a thymus-dependent response [404, 441], to which $CD1^+$ NK T cells partially contribute during malaria infection. However, if the changes in splenic microarchitecture are parasite induced, rather than being a consequence of over-reaction of the host immune response, then it is possible that haemozoin might be the trigger for this. This is unlikely however, as haemozoin deposits remain in the spleen long after clearance of parasitaemia and reformation of the splenic microarchitecture. A study of splenic microarchitecture after administration of the synthetic molecule β-haematin would be informative. Injection of LPS induces some of the same changes in splenic microarchitecture that are observed during malaria infection [349], although with only a single injection these changes are less extensive and of shorter duration. When administered during the acute phase of *P.vinckei* infection, LPS increases the extent of changes in the splenic microarchitecture and enhances the severity of the disease [442]. LPS given after day 7 of a *P.berghei* infection also enhances the severity of disease, however it has a protective effect when administered earlier in infection, and has a varying protective effect (depending on the time of administration) in a non-lethal *P.yoelii* infection [443]. This indicates that changes in the splenic microarchitecture during acute malaria infection may be triggered by activation of TLRs, and that the extent of these changes, as

well as their significance for resistance or susceptibility to infection, may depend on the species of parasite.

In summary, we have detailed the loss of MZ B cells, MZM and MMM from the marginal zone, beginning at day 5 post-infection. We hypothesise that the role of MZM and MMM in malaria infection is either the direct removal of parasites or the removal of apoptotic cells that have phagocytosed parasites, and that MZ B cells may contribute to the large extra-follicular plasma cell response during the acute infection. Whilst these cells may contribute to the clearance of parasites, they are unlikely to be critical for the resolution of a malaria infection. We also hypothesise that the lack of these cells from the marginal zone is a result of downregulation of the LTβ signalling pathway, along with many other alterations in splenic microarchitecture, that may be triggered by the activation of TLRs.

In *P.chabaudi* infection, detection of MAdCAM-1 was used as a marker for the splenic marginal sinus. Its expression spreads to cover the white pulp area in a network staining pattern by day 10 of infection. Despite the extensive changes in splenic microarchitecture, it is interesting in itself that the marginal sinus retains its structure, however the increased expression of MAdCAM-1 over the white pulp area is an intriguing finding. The expression of MAdCAM-1 in the white pulp during infection is similar to the expression pattern seen with macrophage depletion [189]. MAdCAM-1 expression is unchanged in *L.donovani* infection, however, when only MZM are lost from the MZ [172], and may therefore be linked to the presence of MMM. This expression pattern is also seen in mice lacking the sphingosine-1-phosphate lysophospholipid receptor ($S1P_3$) [444], which is required for retention of MZ B cells, MZM and MMM in the marginal zone [437]. Mice that do not express LTβ on B cells also exhibit this network expression pattern of

MAdCAM-1 in the white pulp, indicating that it is a direct effect of lack of signalling through the LTβ signalling pathway. Whether lack of LTβ itself, or lack of signalling downstream of LTβ, is responsible for this expression pattern has, however, not been determined.

The function of MAdCAM-1 in the splenic MZ is unknown, as administration of blocking antibodies *in vivo* has no discernable effect on lymphocyte migration at this site [189]. Increased expression of MAdCAM-1, however, has been suggested to facilitate entry of cells into the B cell follicle [444], and it is possible that MAdCAM-1 expression is induced to aid the reformation of the white pulp, and to guide B and T cells back into their correct locations.

In a study of human spleens, Steiniger *et al.* [183] observe MAdCAM-1 positive cells in the MZ and perifollicular zone, penetrating the white pulp in a reticular pattern, that also stain positive for smooth muscle α-actin. They note MAdCAM-1 positive cells ensheathing bundles of fibres, and postulate that these cells aid $CD4^+$ T cell immigration into the white pulp.

MAdCAM-1 has two known ligands; the integrin α4β7 and L-selectin, which can be expressed on a wide variety of lymphocytes [445, 446]. L-selectin is an adhesion molecule that is expressed on most B cells and naïve T cells, and is upregulated upon activation [447]. However, when increased MAdCAM-1 expression is seen, T cells have migrated out of the white pulp into the red pulp and would therefore not be binding to MAdCAM-1 at this time. Analysis of the expression profile of integrin α4β7 during the acute malaria infection may help to determine a function of MAdCAM-1 in the spleen.

MAdCAM-1 expression in *P.chabaudi* infection is similar to the pattern of splenic conduit staining described by Nolte *et al* [421]. The splenic conduit is a network of reticular fibroblasts in the white pulp of the spleen that is thought to be involved in chemokine signalling and lymphocyte trafficking. It is logical for adhesion molecules to be induced on a network such as the splenic conduit, however MAdCAM-1 expression on the splenic conduit is unproven. Staining for ER-TR7, expressed on fibroblasts of the splenic conduit [421], in our hands did not produce a staining pattern resembling that seen by Nolte *et al.* We do, however, see an increase in expression of this marker in the red pulp. This could be due to either proliferation or expansion of fibroblasts to accommodate the increased volume of the spleen, or induction of expression of ER-TR7 on cells not normally expressing this molecule. ER-TR7 expression is suggested to be controlled by signalling through the lymphotoxin β receptor in combination with the TNF receptors [422], therefore this increase in expression could be related to the alterations in LTβ and TNFα signalling. TNFα alone or in combination with LTα is, however, unable to induce formation of the reticular network incorporating ER-TR7 expression [422], indicating that there may be additional factors controlling expression of this molecule during the acute malaria infection. CD31 (PECAM-1) is another adhesion molecule expressed by endothelial cells in the spleen [423]. As with ER-TR7, expression of this molecule is seen in the red pulp in naïve mice, with minor expression in the T cell zone. CD31 expression increases towards the peak of infection. Expression of CD31 is enhanced by stimulation of the sphingosine-1-phosphate (S_1P) receptors [448]. Marginal zone B cells also express S_1P receptors, and the balance between S_1P and CXCL13 is thought to retain MZ B cells in the marginal zone [437]. In acute *P.chabaudi* infection MZ B cells move out of the marginal zone into the red

pulp, which may be due to decreased expression of CXCL13, but may also (along with increased CD31 expression), be due to increased levels of S_1P. The expression pattern of CD31 is similar to that of ER-TR7 throughout the acute infection, and at no time resembles the expression pattern seen for MAdCAM-1.

Therefore, the identity of the cells on which MAdCAM-1 expression is induced in the white pulp still cannot be determined, however one possibility is that they are gp38-expressing T cell zone stromal cells [449]. We observe an increase in fibroblast and endothelial cell surface molecules in the red pulp as the spleen increases in size, however the location of these cells does not change.

Acute infection with *P.yoelii* induces the formation of "barrier cells", which form a branched complex of cells closing off the splenic filtration beds, protecting haematopoietic cells from infection and compartmentalising the white pulp [347]. Such closing of the splenic circulation has also been described by Krücken *et al* [416] in *P.chabaudi* infection and Yadava *et al* [306] in *P.chabaudi adami* infection, although they do not speculate further as to the cell population which is enabling this closure. Kraal *et al* [189] concluded that the MAdCAM-1 expressing cells were endothelial cells related to those lining the marginal sinus, however it is possible that barrier cells and MAdCAM-1 expressing cells are in fact the same. The origin of barrier cells has not been determined, and cell surface molecules specific to barrier cells have not been described, therefore further work would be required to confirm that barrier cells express MAdCAM-1 during acute *P.chabaudi* infection. Infection of MAdCAM-1 knockout mice will help to determine the significance of this adhesion molecule in malaria infection.

Despite the changes occurring in the splenic microarchitecture, germinal centre formation still occurs. These germinal centres are larger and more numerous than those induced by immunisation [346]. Larger than normal germinal centres can be induced by soluble antigens, and have been shown to have impaired affinity maturation [145]. In *P.chabaudi* infection, a larger proportion of antibodies remain low-affinity after primary infection than in mice immunised with tetanus toxoid [396].

The presence of PNA^+ cells throughout the red pulp between days 7 and 9 post-infection is unexpected. This may indicate some delay in the formation of germinal centres, possibly due to a lack of appropriate chemokines to allow germinal centre formation [156], but does not appear to impede the normal germinal centre functions in any way. Germinal centres are in close association with T cells throughout the acute infection, with some T cells always found inside them. Even during the period of maximum alteration in the microarchitecture, T cells cluster next to germinal centres. Either the association of T cells with germinal centre cells requires different signals to those regulating germinal centre formation or the lack of defined germinal centres around the peak of infection has another, as yet undefined, cause. It would be interesting to determine which malaria antigen germinal centre cells are specific for, however attempts to stain antigen-binding B cells with fluorescently labelled recombinant malaria antigens by histology were unsuccessful (data not shown).

There is also an unusually large number of plasma cells present in the spleen at, and just, after the peak of parasitaemia. The peak number of plasma cells in the spleen occurs at day 10 post infection [346], at the same time as peak numbers of plasma cells are seen after immunisation [450], however the number of plasma cells seen at the peak of the malaria

response is much larger than that seen in immune responses to other infections [231], or after immunisation with non-replicating antigens [346]. The antigen specificity of these plasma cells has yet to be determined, and it is possible that not all of these plasma cells are malaria-specific but may have been non-specifically activated, as polyclonal B cell activation in both human [451] and mouse [452, 453] malaria infection is well documented. In the early stages of infection (up to day 7) most of the splenic plasma cells present are IgM^+. By contrast, the majority of splenic plasma cells seen at later stages of infection (i.e. day 8-20) are IgG^+. This indicates that isotype switching from IgM to IgG production is taking place around day 7-8 of infection. Further detailed analysis (e.g. antigen specificity, IgG isotypes) would be required to fully characterise the malaria-specific B cell response. The characteristic changes occurring in splenic microarchitecture described here and elsewhere [345, 346, 416] point to a disturbance in the lymphotoxin signalling pathway (Figure 7). In the spleen of LTα knockout mice, B and T cell segregation is disturbed, follicular dendritic cells are absent and germinal centres are not formed [454]. This is due to a combination of a lack of signalling through both TNFRI and LTβR (review [29]). Germinal centres are, however, present in LTβ knockout mice. LTα and β knockout mice lack marginal metallophilic macrophages and do not express the adhesion molecule MAdCAM-1 on the sinus endothelium, however mice lacking LTβ solely on B cells have increased MAdCAM-1 expression in a network pattern over the white pulp area [170]. Follicular dendritic cells (FDCs) are absent in mice lacking TNFα, LTα and LTβ alone or in combination [164], which has particular relevance for the development of B cell responses.

Whilst many of the changes in splenic microarchitecture that we observe in acute *P.chabaudi* infection also occur in the absence of LTα, the distinctive changes in MAdCAM-1 expression observed suggest a loss of LTβ rather than LTα signalling. In order to test our hypothesis, that a defect in LTβ signalling is primarily responsible for the alterations in splenic microarchitecture, we administered an agonistic LTβR antibody [455]. Treatment was given at day 4 post-infection, as this is just before changes in splenic microarchitecture become visible, during the period of exponential rise in parasitaemia. Unfortunately a single treatment had no effect on either the primary or secondary infection as determined by parasitaemia and splenic microarchitecture at day 10 post-infection. This does not necessarily indicate that the hypothesis is incorrect, only that the treatment given was insufficient to produce a noticeable effect. Repeated doses at 2 day intervals throughout the acute infection, certainly for the first two weeks, would be more likely to produce a detectable effect. It should also be possible to detect changes in LTβ and CXCR5 on the surface of splenic lymphocytes, and to detect changes in expression of LTβR and CXCL13 by histology [179]. Preliminary experiments have, however, been unsuccessful (data not shown). In viral infection, where similar alterations to the splenic microarchitecture are seen, reduced expression of CCL21 mRNA in the spleen has been described [174].

LTβ is a membrane-bound molecule which binds to the LTβR on stromal cells. This signals through the alternative NF-κB pathway [456, 457] to induce the release of the chemokine CXCL13. CXCL13 and its receptor, CXCR5, have been shown to be crucial for the proper formation of splenic microarchitecture, particularly the formation of B cell follicles [155, 158, 454].

An abundance of TNFα, which has been demonstrated during acute malaria infection [151, 259], may also contribute to the disruption of splenic microarchitecture. TNFα and/or LTα is necessary for CCL19/CCL21 signalling that is responsible for localisation of MZM and FDC, germinal centre formation and MAdCAM-1 expression [164, 179, 181]. An excess of TNFα, however, has been shown to downregulate the expression of these cytokines [172, 179], leading to the loss of MZM.

However, TNFα can also induce the expression of LTβ by signalling through NF-κB [458]. Whether an excess of TNFα downregulates LTβ expression as well as downregulating CCL19/21 expression is not known. Reducing TNFα expression during acute malaria may therefore help to maintain the splenic microarchitecture.

It would also be interesting to determine the cause of this proposed alteration in LTβ signalling. As the β subunit anchors LT to the cell surface, it is possible that a specific block in expression of the β subunit without alteration of expression of the α subunit would by default cause an increase in secretion of soluble LTα. Although many of the immunopathogenic sequelae in acute malaria have been attributed to TNFα, it has recently been proposed that these are in fact more due to LTα [172]. The function of LTα in maintenance of splenic architecture is difficult to investigate as expression of the α subunit is required for LTβ function, and LTα binds the same receptors as TNFα. The expression and regulation of expression of lymphotoxin during acute malaria infection certainly bears further investigation. Likewise, a potential role for the LTβR ligand LIGHT cannot be ruled out, as over-expression of LIGHT has been shown to upregulate expression of MAdCAM-1 in tumour tissue, together with expression of CCL21 [174]. It is also possible

that blockage of the pathway occurs further downstream, at the level of expression of CXCL13, CCL19 and CCL21 or their binding to CXCR5 and CCR7.

Many pathogens, particularly viruses, mediate immune escape by mimicking host ligand and receptor molecules, including those of the TNFR family [459]. It is possible that the parasite expresses a decoy ligand or receptor for LTβ that blocks signalling through this pathway. TNF and lymphotoxin receptors are expressed on stromal cells, which then produce chemokines to attract lymphocytes. Many parasites bind to or infect stromal cells and are therefore in a position to alter the function of these cells [172, 460, 461]. The effect of malaria infection on the splenic stroma has not been investigated, and would be an interesting topic for future work.

Both germinal centre formation and migration of dendritic cells into the spleen are thought to require lymphotoxin-induced chemokine signals [121, 152, 456]. It is, however, difficult to compare data from knockout mice, which have a constitutive lack of signalling, with the temporary alterations we see in acute malaria. Other reports have demonstrated a reduction, but not absence, of germinal centres in $LT\beta^{-/-}$ mice [173, 462], however we do not see a reduction in germinal centres during malaria infection, indicating that either signalling through the LTβ pathway is not completely abrogated during acute malaria infection, that downregulation of the LTβ signalling pathway occurs after germinal centre formation has progressed sufficiently to no longer require this pathway, or that LTβ signalling is not a critical requirement for germinal centre formation. DC migration may be promoted by other chemokines such as MIP-1α/β, which are not dependent on lymphotoxin, although only immature DCs are thought to express the receptors for such signals [463] (review [55]).

We have detailed extensive changes to the splenic microarchitecture during the acute malaria infection, and have speculated as to the mechanisms that cause these changes. It has been hypothesised that such dramatic changes cannot be advantageous to the host, and are likely to have a detrimental effect on the generation of an immune response and/or memory responses [348]. The fact that similar alterations are found in fatal cases of *P.falciparum* [348] would seem to indicate that such changes are detrimental, although in human infections it is possible that the splenic microarchitecture was altered prior to infection with malaria. Despite these alterations in the splenic microarchitecture, clearance of parasites still occurs in both human and mouse infections. These changes may therefore be required for closure of the splenic circulation to protect the haematopoietic beds of the mouse spleen from infection [347, 416]. Assuming that the changes in splenic microarchitecture are caused by downregulation of the LTβ signalling pathway, a protective effect of these changes is corroborated by the fact that $LT\beta R^{-/-}$ mice were less susceptible to malaria infection than wild-type mice [416]. Further work is required to unravel the contribution and significance of these changes to the pathogenesis of malarial disease.

Chapter 5

Kinetics and lifespan of B cells and plasma cells in the spleen and bone marrow during malaria infection

Introduction

There is a large body of evidence, both in animal and human studies, to show that B cells and antibodies are required for clearance of a primary malaria infection and are important for immunity to subsequent infections (review [262]). μMT mice, which lack B cells, are capable of controlling acute *P.chabaudi* infection, but develop a chronic relapsing parasitaemia [255, 287, 318], and B cell deficient J_HD mice control an acute *P.chabaudi* or *P.vinckei* infection, but not a *P.yoelii* 17X infection [319].

Passive transfer of total serum antibodies from immune mice is at least partially protective in *P.berghei* infection [464-466], *P.chabaudi* infection [466], *P.vinckei* infection [258], and *P.yoelii* infection [99]. Transfer of immune serum is more fully protective in *P.falciparum* infection of squirrel monkeys [467, 468], and transfer of malaria-specific antibodies is protective against *P.yoelii* infection [327].

The strongest evidence that antibodies are important in human malaria infections comes from studies on the protective effect of passive transfer of serum antibodies [320, 321] (review [322, 323]). Antibody responses to malaria antigens in humans are detectable after the first infection [303, 304], and can be associated with resistance to clinical disease [128,

184, 293, 305-312] (review [262]). IgG1 and IgG3 antibodies in particular have been shown to be the most important isotypes for resistance to disease [324, 325]. The presence of anti-malaria antibodies can, however, also be evidence of recent or ongoing infection [294, 311, 361, 469]. Unfortunately, many antibodies do not last long after the end of the transmission season and are therefore unable to protect against reinfection [177, 311, 313, 314, 470-472].

In addition to malaria-specific antibody responses, hypergammaglobulinaemia, which can significantly shorten the half-life of antibodies [473], occurs in both human [451, 474] and mouse [475, 476] malaria infections, and B cell hyperplasia has also been described in both human [451, 477] and mouse malaria infections with *P.chabaudi* [452, 453] and *P.yoelii* [478], particularly in primary infection [452]. The relationship of these large B cell and plasma cell responses to malaria-specific antibody production is unclear, however polyclonal B cell activation and hypergammaglobulinaemia have been found in other infections where B cell dysfunction occurs [479, 480], and in other parasitic infections (review [477, 481]), suggesting that there may be some dysfunction in the B cell and plasma cell response to malaria. Polyclonal B cell activation in *T.cruzi* infection has been shown to be triggered by a parasite antigen, glutamate dehydrogenase, dependent on production of B cell stimulatory cytokines by $CD11b^+$ cells [482], indicating that such responses may be beneficial for the parasite.

Malaria infection produces a good IgG antibody response in mice to both malaria parasite lysate and recombinant malaria proteins [265, 377, 396, 483, 484], however there is also some production of malaria non-specific plasma cells [452, 453]. In *P.chabaudi* infection, antibodies to malaria antigens drop away quickly as the parasitaemia is cleared.

Large numbers of plasma cells are produced in the spleen during acute malaria infection (Figure 37), far more than are produced in response to immunisation with chicken gamma globulin, however these plasma cells do not seem to persist long in the spleen [346]. Few plasma cells can be supported long-term by the spleen, most plasma cells must migrate to the bone marrow in order to become long-lived cells [148, 228]. The existence of long-lived plasma cells in malaria infection has not been established, and the antigen specificity of the large number of splenic plasma cells formed during an acute malaria infection is also unknown. From immunohistological studies it is clear that most of the plasma cells formed during acute infection are gone from the spleen within days [346]. It is likely that most of these plasma cells are short-lived, but some may migrate to the bone marrow to become long-lived plasma cells.

Very little is known about the generation of memory B cells in response to malaria infection in either mice or humans. This is partly because a cell surface marker for memory B cells has only recently been identified in humans [237, 485], and not yet identified in mice. Memory B cells are persistent, long-lived, antigen specific B cells that do not secrete antibodies, but can rapidly differentiate into antibody-secreting cells upon secondary antigen encounter (review [486]). They are mostly, but not always, isotype switched cells that have usually undergone somatic hypermutation [487, 488]. Recent work has shown that many people with antibodies to malaria antigens do not have detectable numbers of memory B cells in the blood [400]. Other work in *P.yoelii* infection [367] has shown that memory B cells and long-lived plasma cells induced by immunisation with *P.yoelii* MSP-1_{19}, were ablated by subsequent infection with *P.yoelii*.

This chapter investigates the kinetics of B cell and plasma cell formation in the spleen and bone marrow, using BrdU labelling to follow cells that divide at specific times during the course of infection. In addition, attempts to track malaria antigen specific B cells and plasma cells by flow cytometry using fluorescently labelled recombinant proteins are detailed. Merozoite surface protein-1 (MSP-1) is a leading candidate for a vaccine against the erythrocytic stage of the life cycle (review [339]). It is expressed on the surface of the merozoite as a protein complex, which is cleaved off at invasion, leaving a 19kDa fragment ($MSP\text{-}1_{19}$) on the surface of the invading parasite (review [489]). Antibodies to this fragment can protect against parasite invasion [490]. Although the sequence of $MSP\text{-}1_{19}$ is highly conserved across strains and species, the best protection is still against challenge with the homologous strain. The methods used to fluorescently label MSP-1 and detect MSP-1 binding cells, degree of success and future directions for investigating malaria-specific B cells are discussed.

Results

Establishment of parameters for FACS analysis

The spleen increases several-fold in size during acute malaria infection [184, 345]. The number of lymphocytes in the spleen increases, as does haematopoiesis and apoptosis [184, 342-344, 346, 392]. Far less is known about changes in the cellular composition of the bone marrow during malaria infection, as most studies to date have concentrated on erythropoietic populations, although decreases in cellularity have been described [342-344]. Both the forward light scatter (FSC) and side scatter (SSC) FACS profiles of spleen and bone marrow alter during acute malaria infection, but return to a profile similar to that of naïve mice by approximately 12 weeks after primary infection (Figure 40). The accumulation of parasite pigment, parasite debris and apoptotic material contributes to an increase in autofluorescence in infected spleens and increases the tendency of cells to form clumps in suspension, which can interfere with FACS analysis (unpublished observations). The first priority was therefore to establish the gates and data analysis techniques to be used, and to eliminate dead and autofluorescent cells from the analysis. The live cell gate was set to include lymphocytes and blasted cells twice the size of resting lymphocytes, and to eliminate dead, autofluorescent and large non-lymphoid cells. Approximately 80% of B cells and plasma cells were contained within these gates.

Once the appropriate live cell gate had been established, the problem of autofluorescence was addressed. Unstained samples of both spleen and bone marrow cells autofluoresce in channels 1, 2 and 3. This produces a characteristic diagonal streak in two colour plots (Figure 41A) that cannot be removed by altering the live cell gate, as autofluorescent cells

Figure 40. Selection of the live cell gate for flow cytometric analysis of spleen and bone marrow cells during primary *P.chabaudi* infection.

Forward light scatter (FSC) and side scatter (SSC) of single cell suspensions from spleens (left panel) and bone marrow (right panel) of naïve mice and mice 2, 4, 6, 8 and 12 weeks post-infection are shown as density plots.

A live cell gate including cells with a lymphoid footprint was selected (–). A total of 1 x 10^6 cells were analysed, of which approximately 70-90% were contained within the live cell gate. The percentage of cells contained within the live cell gate is shown on each density plot. These plots are from representative mice (at least 3 mice per time point).

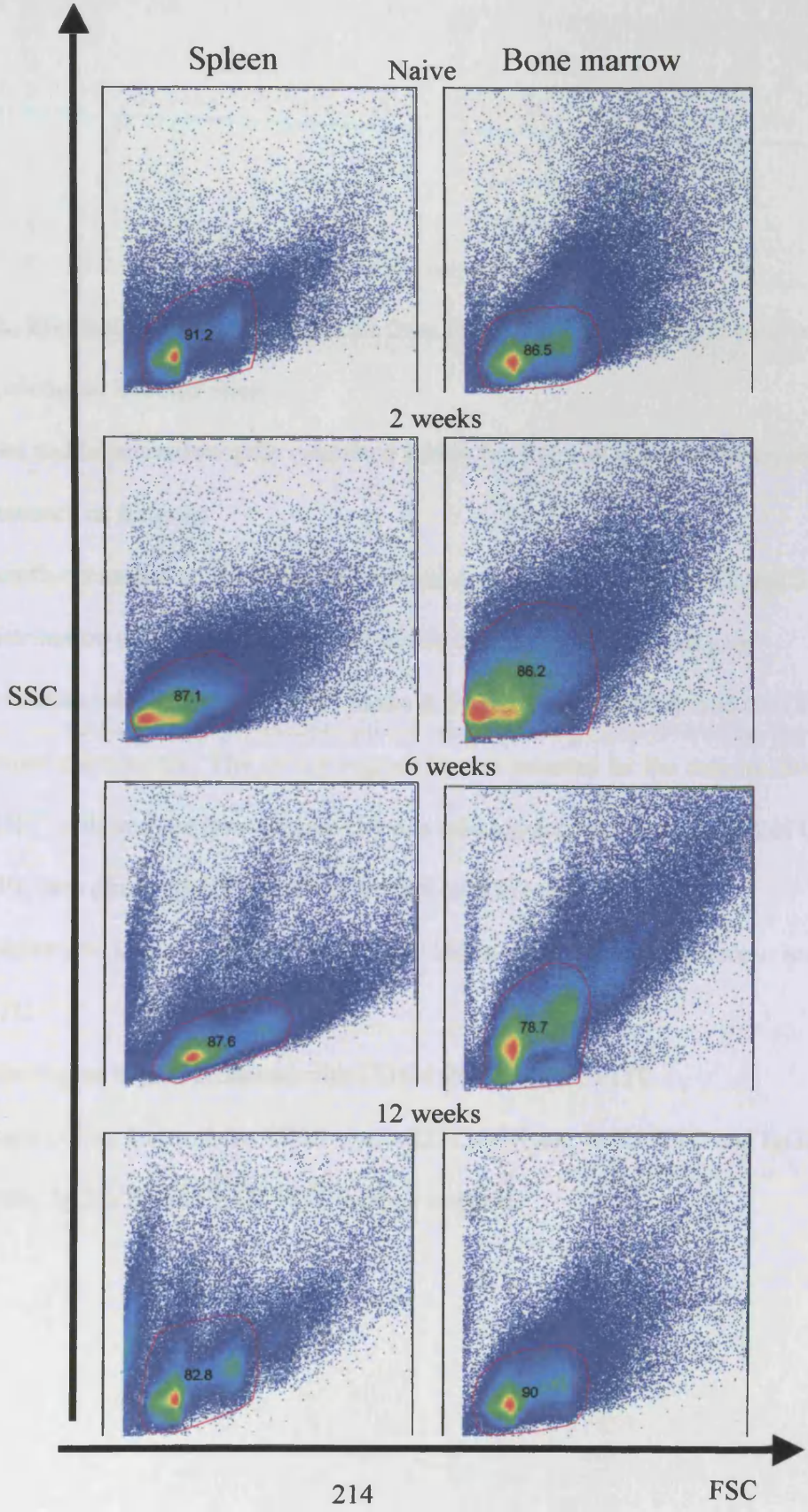

Spleen
Naive
Bone marrow
91.2
86.5
2 weeks
87.1
86.2
6 weeks
87.6
78.7
12 weeks
82.8
90
SSC
214
FSC

Figure 41. Elimination of autofluorescence from the analysis of spleen and bone marrow cells of *P.chabaudi* infected mice

Splenocytes and bone marrow cells contained within the live cell gate were analysed for autofluorescence as follows:

A) Autofluorescence of unstained splenocytes detectable in channels 1, 2 and 3

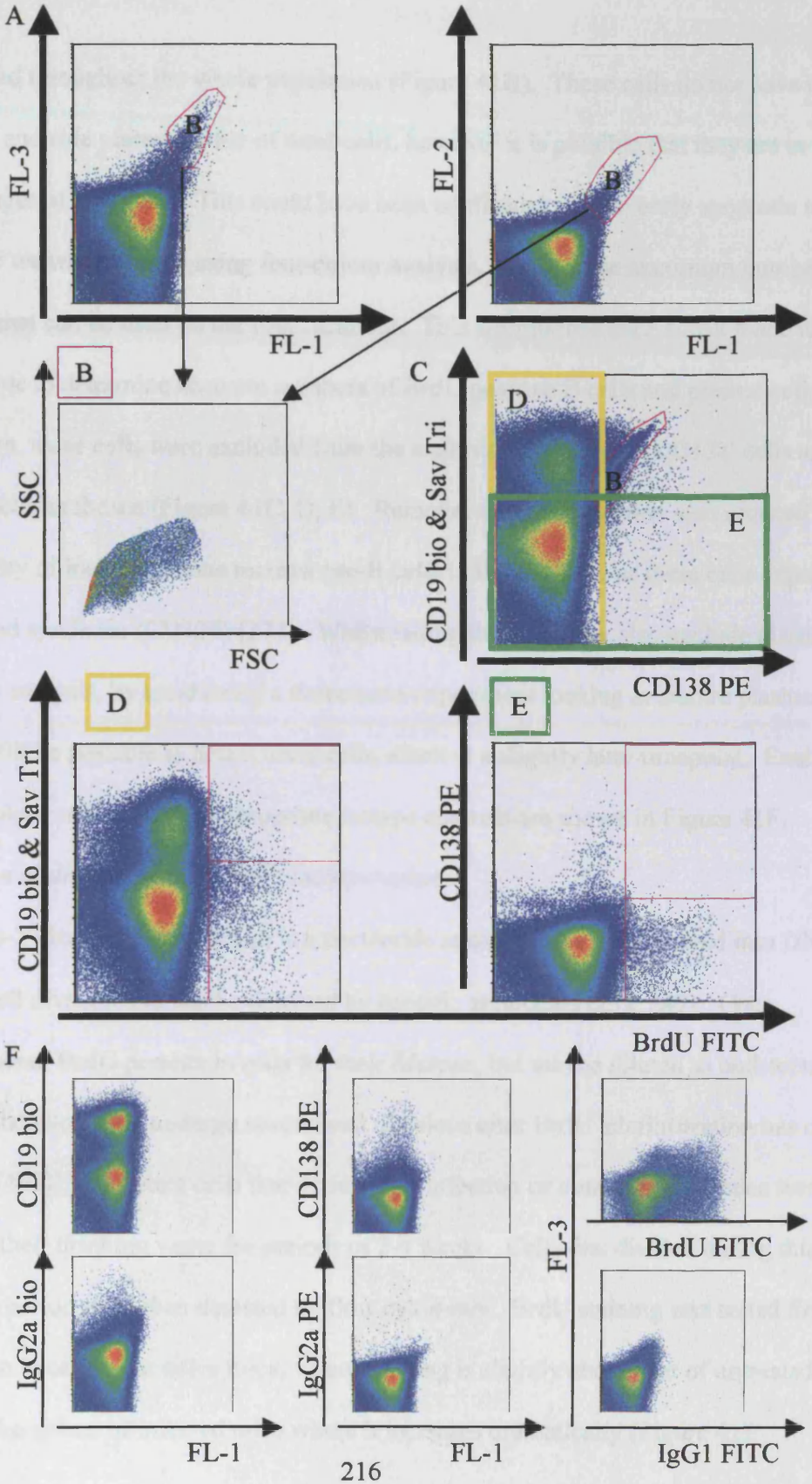
B) Distribution of autofluorescent cells within the FSC, SSC live cell gate

C) Two colour density plot of CD19 biotin & Streptavidin Tricolour and CD138 PE stained splenocytes. The yellow region (D) was selected for the determination of $CD19^+$ cells and the green region (E) was selected for the determination of $CD138^+$ cells, thus eliminating the autofluorescent cells in region B

D) Splenocytes in gate D stained with CD19 biotin & Streptavidin Tricolour and BrdU FITC

E) Splenocytes in gate E stained with CD138 PE and BrdU FITC

F) Single colour controls for CD19 biotin, CD138 PE and BrdU FITC and IgG2a biotin, IgG2a PE and IgG1 FITC isotype controls


A
FL-3
FL-1
B
FL-2
FL-1
B
B
SSC
FSC
C
CD19 bio & Sav Tri
CD138 PE
D
B
E
D
CD19 bio & Sav Tri
E
CD138 PE
BrdU FITC
F
CD19 bio
IgG2a bio
FL-1
CD138 PE
IgG2a PE
FL-1
FL-3
BrdU FITC
IgG1 FITC

are spread throughout the whole population (Figure 41B). These cells do not have the forward and side scatter profile of dead cells, however it is possible that they are in the early stages of apoptosis. This could have been confirmed with an early apoptotic marker, however we were already using four-colour analysis, which is the maximum number of colours that can be used on the FACSCalibur. This autofluorescence streak made it impossible to determine accurate numbers of BrdU positive B cells and plasma cells. Therefore, these cells were excluded from the analysis, by gating on $CD138^-$ cells and $CD19^-$ cells as shown (Figure 41C, D, E). Removal of this population also reduced the probability of including bone marrow pre-B cells in the analysis, as these cells express both CD19 and syndecan (CD138) [373]. Whilst taking this step may also exclude plasmablasts from the analysis, by conducting a timecourse experiment looking at mature plasma cells it should still be possible to detect these cells, albeit at a slightly later timepoint. Examples of single colour stains and the appropriate isotype controls are shown in Figure 41F.

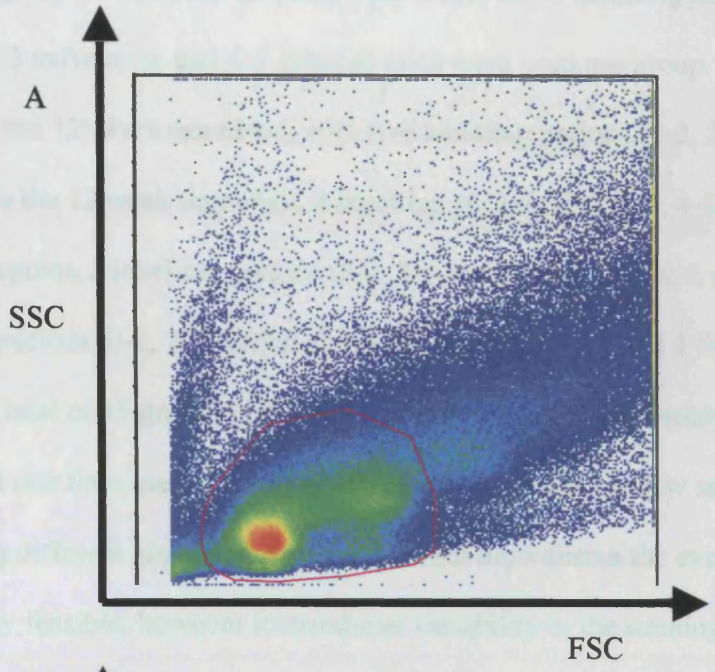
Detection of divided cells by BrdU incorporation

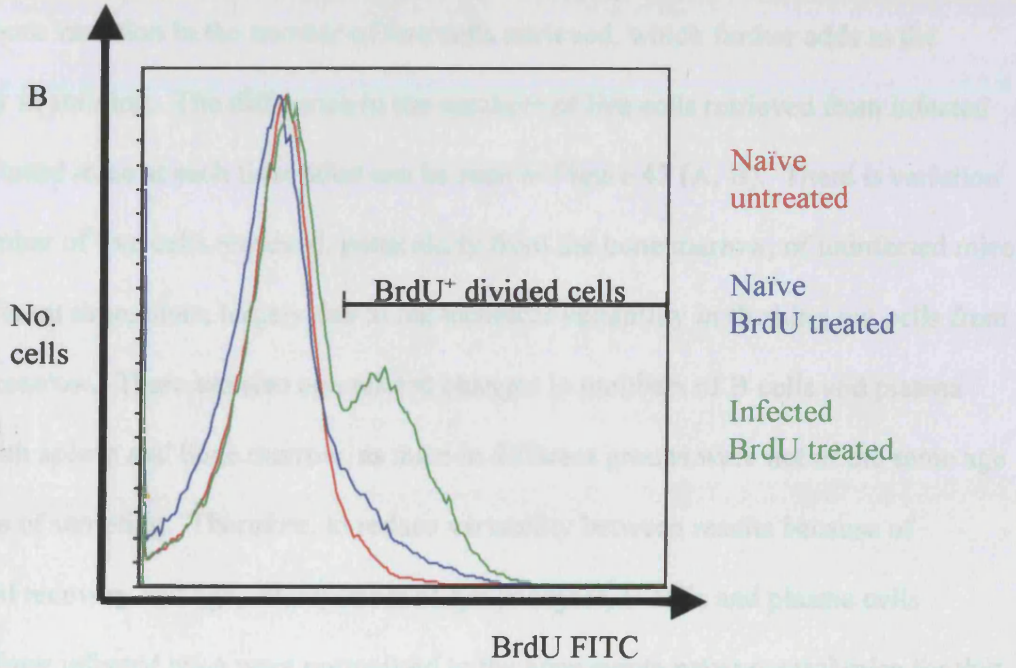
5-Bromo-2'-deoxyuridine (BrdU) is a nucleotide analog that is incorporated into DNA during cell division and can be detected by specific antibodies [232, 247]. Once incorporated, BrdU persists in cells for their lifespan, but maybe diluted to undetectable levels if labelled cells undergo several cell divisions after BrdU administration has ceased (review [491]). To detect cells that divide after infection or immunisation, mice were given BrdU in their drinking water for periods of 2-4 weeks. Cells that divided during this labelling period were then detected by flow cytometry. BrdU staining was tested first on the spleen in otherwise naïve mice, where staining is slightly above that of untreated mice, then on the spleen of infected mice where it increases dramatically (Figure 42).

Figure 42. Cell division in *P.chabaudi* infected mice detected by incorporation of BrdU Spleens were taken from mice that were not BrdU treated, mice treated with BrdU for 37 days and mice treated with BrdU for the first 7 days of malaria infection.

A) FSC SSC profile indicating the live cell gate (–)

B) Histogram of fluorescence intensity of BrdU staining in untreated (–), BrdU treated (–) and infected mice (–)


A
SSC
FSC


B
No. cells
BrdU+ divided cells
Naïve untreated
Naïve BrdU treated
Infected BrdU treated
BrdU FITC

Normalisation of results

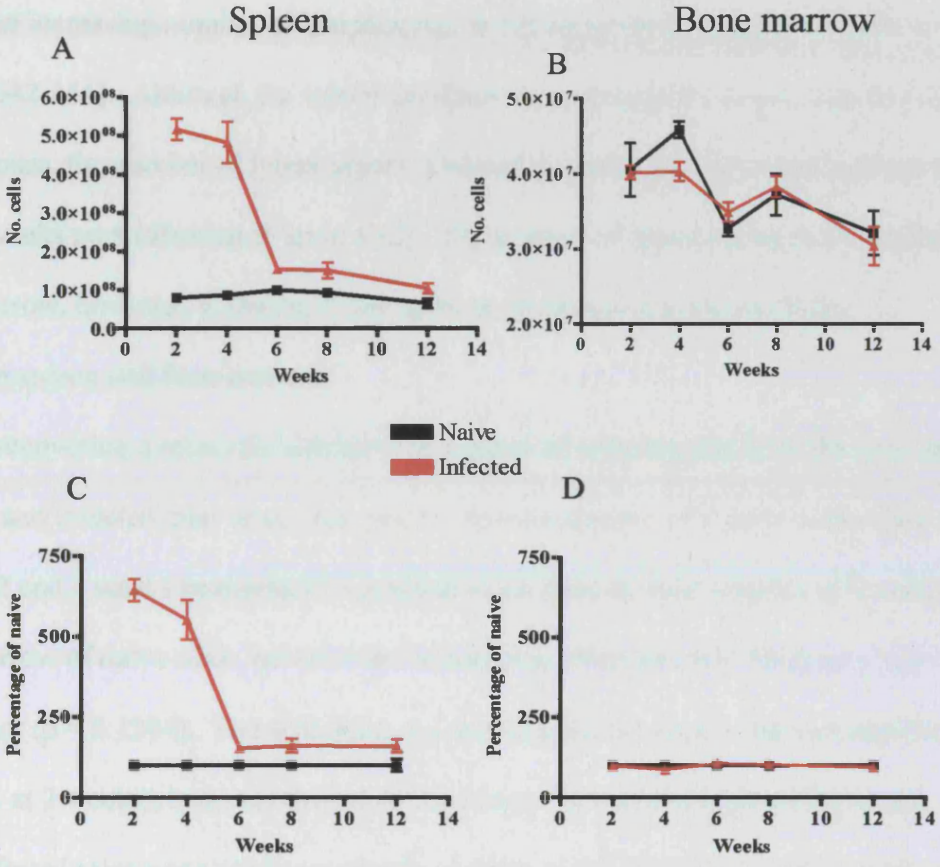
For each group of mice, representing a particular BrdU labelling period and timepoint post-infection, 3 naïve mice and 4-5 infected mice were used per group. Five timepoints (weeks 2, 4, 6, 8 and 12) were examined, with five labelling periods (0-2, 2-4, 4-6, 6-8 and 8-12 weeks) for the 12 week timepoint, 4 labelling periods (0-2, 2-4, 4-6, 6-8 weeks) for the 8 week timepoint, 3 labelling periods (0-2, 2-4, 4-6 weeks) for the 6 week timepoint, 2 labelling periods (0-2, 2-4 weeks) for the 4 week timepoint and 1 for the 2 week timepoint, making a total of 15 groups (120 mice). It was not possible to analyse such a large number of mice at one time, particularly as both spleen and bone marrow samples were taken. Analysing different groups of mice on different days allows the experiment to be technically feasible, however it introduces variability in the staining. In the bone marrow there is more variation in the number of live cells retrieved, which further adds to the variability in staining. The difference in the numbers of live cells retrieved from infected and uninfected mice at each time point can be seen in Figure 43 (A, B). There is variation in the number of live cells retrieved, particularly from the bone marrow, of uninfected mice at the different timepoints, largely due to the technical variability in flushing out cells from the bone marrow. There are also age-related changes in numbers of B cells and plasma cells in both spleen and bone marrow, as mice in different groups were not of the same age at the time of sampling. Therefore, to reduce variability between results because of differential recovery and age, total number of lymphocytes, B cells and plasma cells obtained from infected mice were normalised to the appropriate naïve control mice for that time point. (Figure 43 C, D). All subsequent figures show cell numbers expressed as a percentage of cell numbers in naïve mice per spleen or per two femurs (12.6% of total

Figure 43. Normalisation of live cell numbers in spleens and bone marrow of *P.chabaudi* infected mice

The cell numbers shown the means and SEM total numbers of lymphocytes retrieved from spleens and bone marrow (2 femurs). Total lymphocyte numbers from each BrdU labelling period were pooled for each timepoint, therefore live cell numbers are from a minimum of 3 mice (week 2, naïve) and a maximum of 25 mice (week 12, infected)

A) and B) show a comparison between numbers of lymphocytes from naïve (–) and infected (–) mice in the spleen (A) and bone marrow (B) taken at different time points post-infection with age matched naïve controls.

C) and D) show numbers of lymphocytes from infected mice normalised to the naïve controls for each time point (absolute cell number for spleen and 2 femurs expressed as a percentage of naïve controls) in spleen (C) and bone marrow (D)


Spleen
Bone marrow
A
B
No. cells
Weeks
Naive
Infected
C
D
Percentage of naive
Weeks

mouse bone marrow is located in two femurs [492]). Where low numbers of cells meanthat this normalisation skews the results, absolute cell numbers are also shown as a comparison. The spleen increases around 7-fold in size during the acute infection, gradually diminishing in size as the infection becomes chronic (Figure 43C). The increase in spleen size is due both to an increasing number of lymphocytes and to an increase in erythropoiesis in the spleen [342-344]. Although the spleen itself remains permanently larger than that of a naïve mouse, the number of lymphocytes retrieved is similar for naïve and infected spleens from 6 weeks post-infection (Figure 43C). The number of lymphocytes retrieved from bone marrow, however, is similar to the naïve at all timepoints (Figure 43D).

B cells in spleen and bone marrow

Despite recovering a relatively similar total number of lymphocytes from the bone marrow of naïve and infected mice at all time points, the total number of B cells in the bone marrow of mice 2 and 4 weeks post-infection is much lower than the total number of B cells in the bone marrow of naïve mice, however at 4 weeks post-infection, this difference is not significant ($p = 0.1564$). The difference at 2 weeks post-infection is also not significant ($p = 0.0571$ at 2 weeks), however the test at this timepoint is not very powerful as only 3 naïve and 4 infected mice were used, compared to 6 naïve and 9 infected mice at 4 weeks post-infection. The total number of B cells in the bone marrow does not fully return to normal levels until 6 weeks post-infection (Figure 44A). This may be because of increased export of B cells into the circulation, and to other lymphoid organs such as the spleen. In line with this possibility, there are approximately 50% more B cells in the spleen at 2 weeks post-infection than in naïve mice.

The number of B cells in the spleen continues to increase up to 4 weeks post-infection,

Figure 44. Reduction of B cell numbers in the bone marrow at 2 weeks post-infection

A) Total numbers of $CD19^+$ B cells in spleen (–) and bone marrow (--) relative to naïve controls (–)

B) Experimental plan to show the period of BrdU labelling ( ) and sampling time points (↓) for spleen and bone marrow during infection

C) Total numbers of BrdU labelled $CD19^+$ B cells that divided in the first two weeks of infection in spleen (–) and bone marrow (--) relative to naïve controls (–)

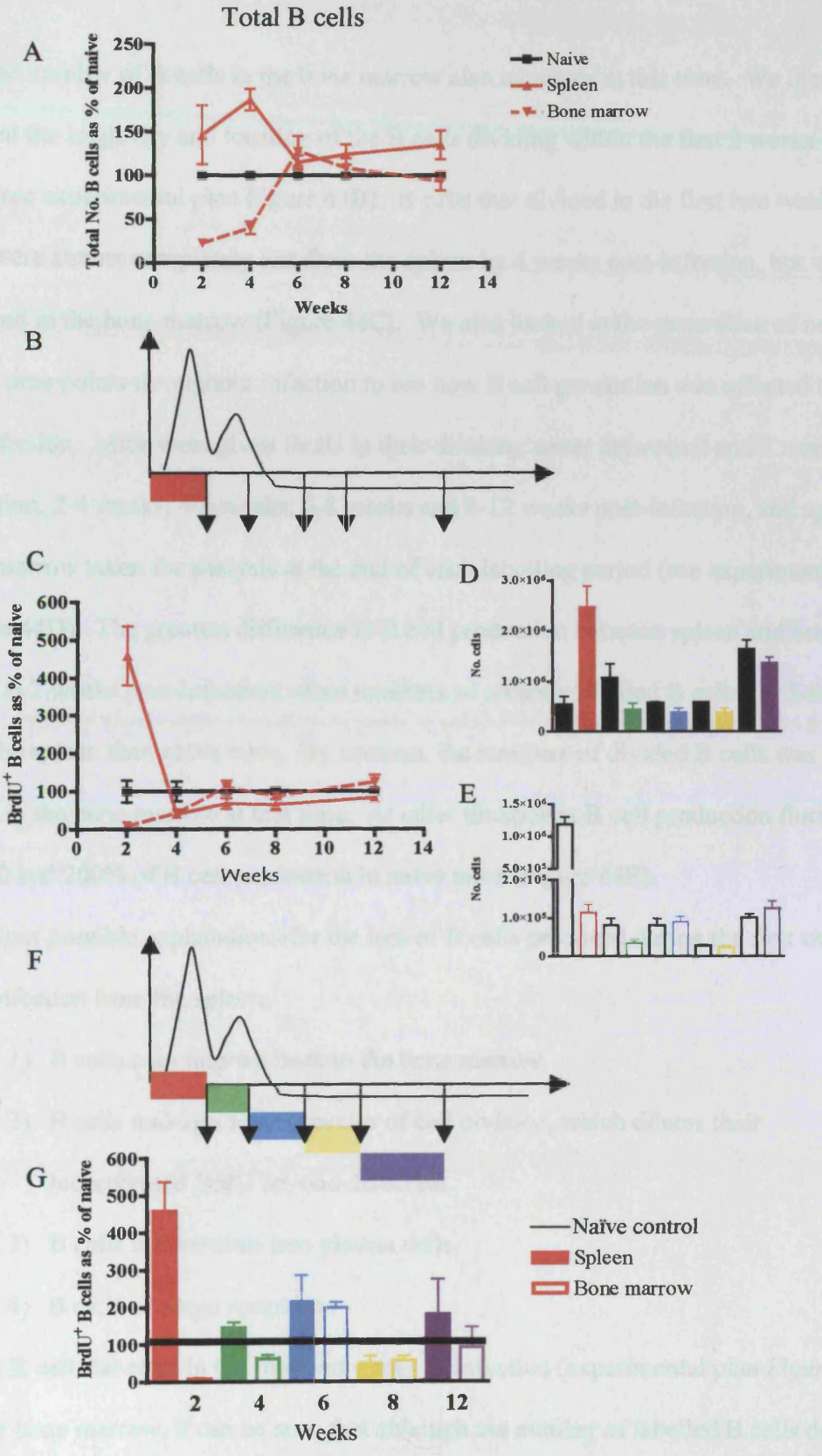
D) Absolute numbers of BrdU labelled B cells in naïve (■) and infected (■) spleen

E) Absolute numbers of BrdU labelled B cells in naïve (□) and infected (□) bone marrow (2 femurs)

F) Experimental plan to show period of BrdU labelling; 0-2 weeks ( ), 2-4 weeks ( ), 4-6 weeks ( ), 6-8 weeks ( ) & 8-12 weeks ( ) post-infection; and sampling time points (↓) for spleen and bone marrow during infection

G) Kinetics of B cell incorporation of BrdU in spleen (■) and bone marrow (□) throughout acute and chronic infection

Points and bars represent the mean value +/- SEM of 3 age matched naïve controls and 4-5 infected mice per time point, and the horizontal black line indicates the normalised naïve controls (100%)


Total B cells
A
Total No. B cells as % of naive
250
200
150
100
50
0
0 2 4 6 8 10 12 14
Weeks
Naive
Spleen
Bone marrow
B
C
BrdU+ B cells as % of naive
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14
Weeks
D
No. cells
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
E
No. cells
1.5×10⁶
1.0×10⁶
5.0×10⁵
2.0×10⁵
1.0×10⁵
0
F
G
BrdU+ B cells as % of naive
600
500
400
300
200
100
0
2 4 6 8 12
Weeks
Naïve control
Spleen
Bone marrow

however the number of B cells in the bone marrow also increases at this time. We therefore investigated the longevity and location of the B cells dividing within the first 2 weeks of infection (see experimental plan Figure 44B). B cells that divided in the first two weeks of infection were almost completely lost from the spleen by 4 weeks post-infection, but were not observed in the bone marrow (Figure 44C). We also looked at the generation of new B cells at all time points throughout infection to see how B cell generation was affected by malaria infection. Mice were given BrdU in their drinking water between 0 and 2 weeks post-infection, 2-4 weeks, 4-6 weeks, 6-8 weeks and 8-12 weeks post-infection, and spleens and bone marrow taken for analysis at the end of each labelling period (see experimental plan Figure 44D). The greatest difference in B cell production between spleen and bone marrow is at 2 weeks post-infection, when numbers of recently divided B cells are 5-fold higher in the spleen than naïve mice. By contrast, the numbers of divided B cells was 10-fold lower in the bone marrow at that time. At other timepoints B cell production fluctuates between 50 and 200% of B cell production in naïve mice (Figure 44E).

There are four possible explanations for the loss of B cells produced during the first two weeks of infection from the spleen.

1) B cells may migrate back to the bone marrow.
2) B cells undergo several cycles of cell division, which dilutes their incorporated BrdU beyond detection.
3) B cells differentiate into plasma cells.
4) B cells undergo apoptosis.

Looking at B cells labelled in the first two weeks of infection (experimental plan Figure 45A) in the bone marrow, it can be seen that although the number of labelled B cells does

Figure 45. The majority of B cells lost between 2 and 4 weeks post-infection do not migrate to the bone marrow and do not dilute their BrdU label through cell division

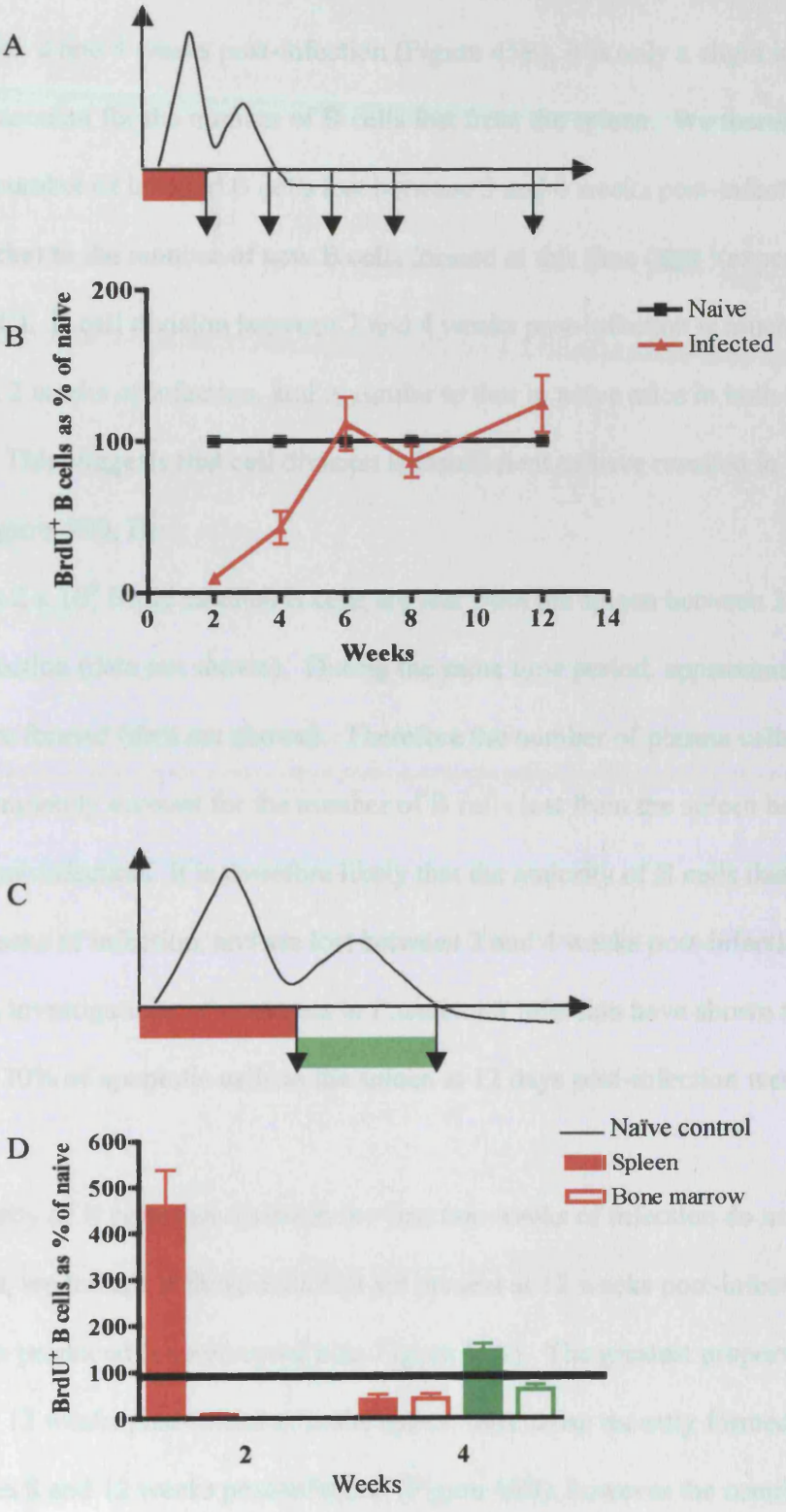
A) Experimental plan to show the period of BrdU labelling ( ) and sampling time points ( ) during infection

B) Total number of BrdU labelled B cells in bone marrow (–) relative to naïve controls (–)

C) Experimental plan to show the period of BrdU labelling; 0-2 weeks ( ), 2-4 weeks ( ) post-infection; and sampling time points ( ) for spleen and bone marrow during infection

D) Total number of BrdU labelled B cells in spleen ( ) and bone marrow ( ) relative to naïve controls (–)

Points and bars represent the mean value +/- SEM of 3 age matched naïve controls and 4-5 infected mice per time point, and the horizontal black line indicates the normalised naïve controls (100%)


A
B
200
100
0
BrdU+ B cells as % of naive
0
2
4
6
8
10
12
14
Weeks
Naive
Infected
C
D
600
500
400
300
200
100
0
BrdU+ B cells as % of naive
2
4
Weeks
Naïve control
Spleen
Bone marrow

increase between 2 and 4 weeks post-infection (Figure 45B), it is only a slight increase and insufficient to account for the number of B cells lost from the spleen. We therefore compared the number of labelled B cells lost between 2 and 4 weeks post-infection (2 weeks to 4 weeks) to the number of new B cells formed at this time ( )(experimental plan Figure 45C). B cell division between 2 and 4 weeks post-infection is much less than during the first 2 weeks of infection, and is similar to that in naïve mice in both spleen and bone marrow. This suggests that cell division is insufficient to have resulted in loss of the BrdU label (Figure 45D, E).

Approximately 2 x 10^6 BrdU labelled B cells are lost from the spleen between 2 and 4 weeks post infection (data not shown). During the same time period, approximately 2 x 10^5 plasma cells are formed (data not shown). Therefore the number of plasma cells produced also cannot completely account for the number of B cells lost from the spleen between 2 and 4 weeks post-infection. It is therefore likely that the majority of B cells that divided in the first two weeks of infection, and are lost between 2 and 4 weeks post-infection, have died. Previous investigations of apoptosis in *P.chabaudi* infection have shown that approximately 30% of apoptotic cells in the spleen at 12 days post-infection were B cells [392].

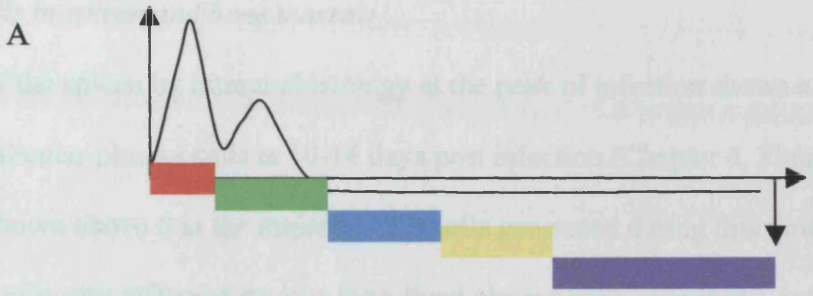
Since the majority of B cells that divide in the first two weeks of infection do not become long-lived cells, we looked at those cells that are present at 12 weeks post-infection to see when they were produced (experimental plan Figure 46A). The greatest proportion of B cells present at 12 weeks post-infection in the spleen were those recently formed, i.e. divided between 8 and 12 weeks post-infection (Figure 46B), however the contributions from cells dividing at all time points except 2-4 weeks post-infection were similar to each

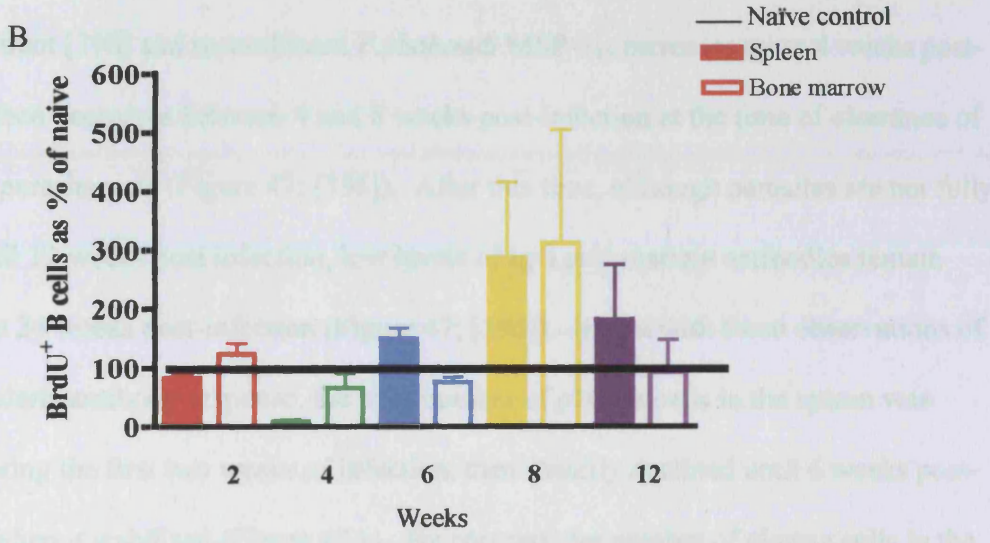
Figure 46. B cells present at 12 weeks post-infection are mostly recently produced cells

A) Experimental plan to show the period of BrdU labelling; 0-2 weeks ( ), 2-4 weeks ( ), 4-6 weeks ( ), 6-8 weeks ( ) & 8-12 weeks ( ) post-infection; and sampling time point (↓) for spleen and bone marrow at 12 weeks post-infection

B) Total number of BrdU labelled B cells in spleen (■) and bone marrow (□) relative to naïve controls (–)

Points and bars represent the mean value +/- SEM of 3 age matched naïve controls and 4-5 infected mice per time point, and the horizontal black line indicates the normalised naïve controls (100%)


A


B
Naïve control
Spleen
Bone marrow
BrdU+ B cells as % of naive
600
500
400
300
200
100
0
2
4
6
8
12
Weeks

other. In the bone marrow, the contribution of cells dividing at different time points is always similar to that in naïve mice (Figure 46B), although the greatest absolute number of B cells were those that divided between 8 and 12 weeks post-infection (data not shown).

Plasma cells in spleen and bone marrow

Analysis of the spleen by immunohistology at the peak of infection shows a large number of extra-follicular plasma cells at 10-14 days post infection (Chapter 4, Figure 37; [346]. We have shown above that the majority of B cells generated during this time probably die, and only a minority differentiate into long-lived plasma cells. Similarly, anti-malaria antibody titres contain a large short-lived component; the IgG response to both *P.chabaudi* parasite extract [396] and recombinant *P.chabaudi* MSP-1_{19} increases up to 4 weeks post-infection, then decreases between 4 and 8 weeks post-infection at the time of clearance of detectable parasitaemia (Figure 47; [396]). After this time, although parasites are not fully cleared until 12 weeks post infection, low levels of IgG anti-malaria antibodies remain stable up to 24 weeks post-infection (Figure 47; [396]). In line with these observations of the anti-malaria antibody response, the total number of plasma cells in the spleen was greatest during the first two weeks of infection, then steadily declined until 6 weeks post-infection, when it stabilised (Figure 48A). By contrast, the number of plasma cells in the bone marrow was similar to that in naïve mice at all time points.

The majority of plasma cells lost from the spleen between 2 and 4 weeks post-infection did not appear in the bone marrow (Figure 48C), indicating that these are short-lived cells. Plasma cells are terminally differentiated cells that do not undergo further cell division [493], therefore the loss of BrdU labelled cells from the spleen can only be due to either migration or cell death.

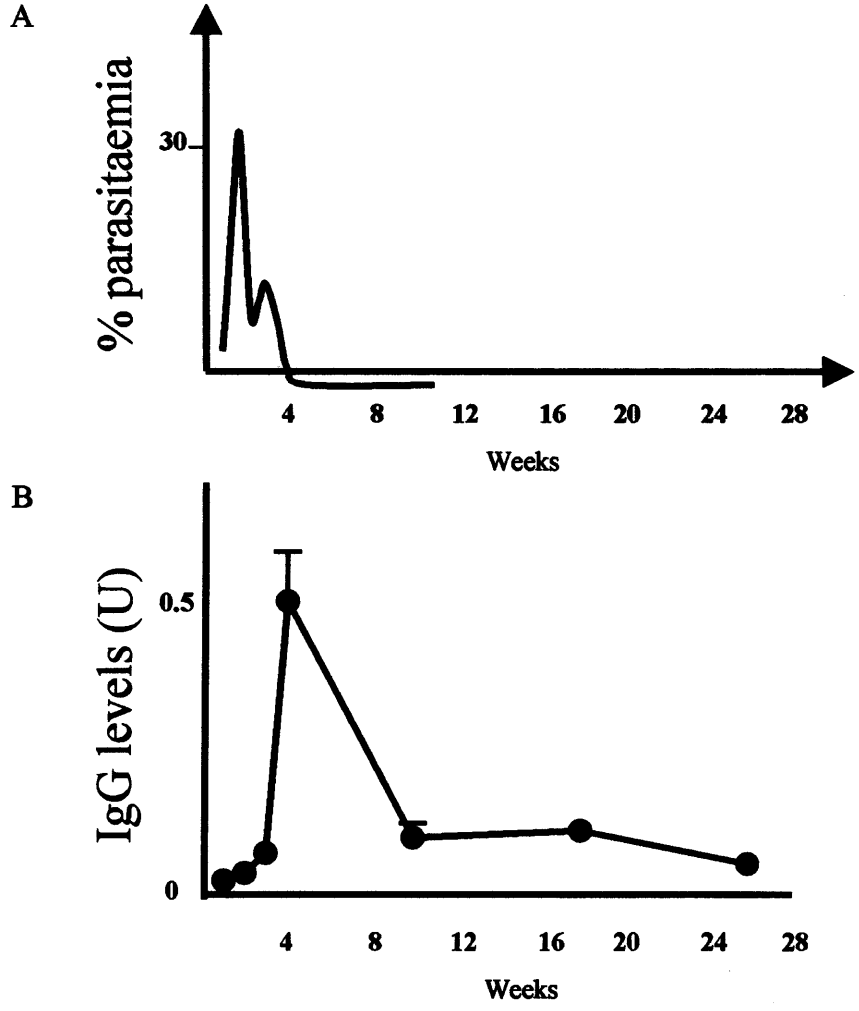


Figure 47. Parasitaemia and anti-MSP-1_{19} IgG antibody titres during primary *P.chabaudi* infection

A) Diagrammatic representation of primary *P.chabaudi* infection

B) Anti-MSP-1_{19} IgG antibody during primary *P.chabaudi* infection

Points represent mean value and SEM of 4-8 mice per timepoint

Figure 48. Majority of plasma cells produced during *P.chabaudi* infection are short lived

A) Total number of plasma cells in spleen (–) and bone marrow (--) relative to naïve controls

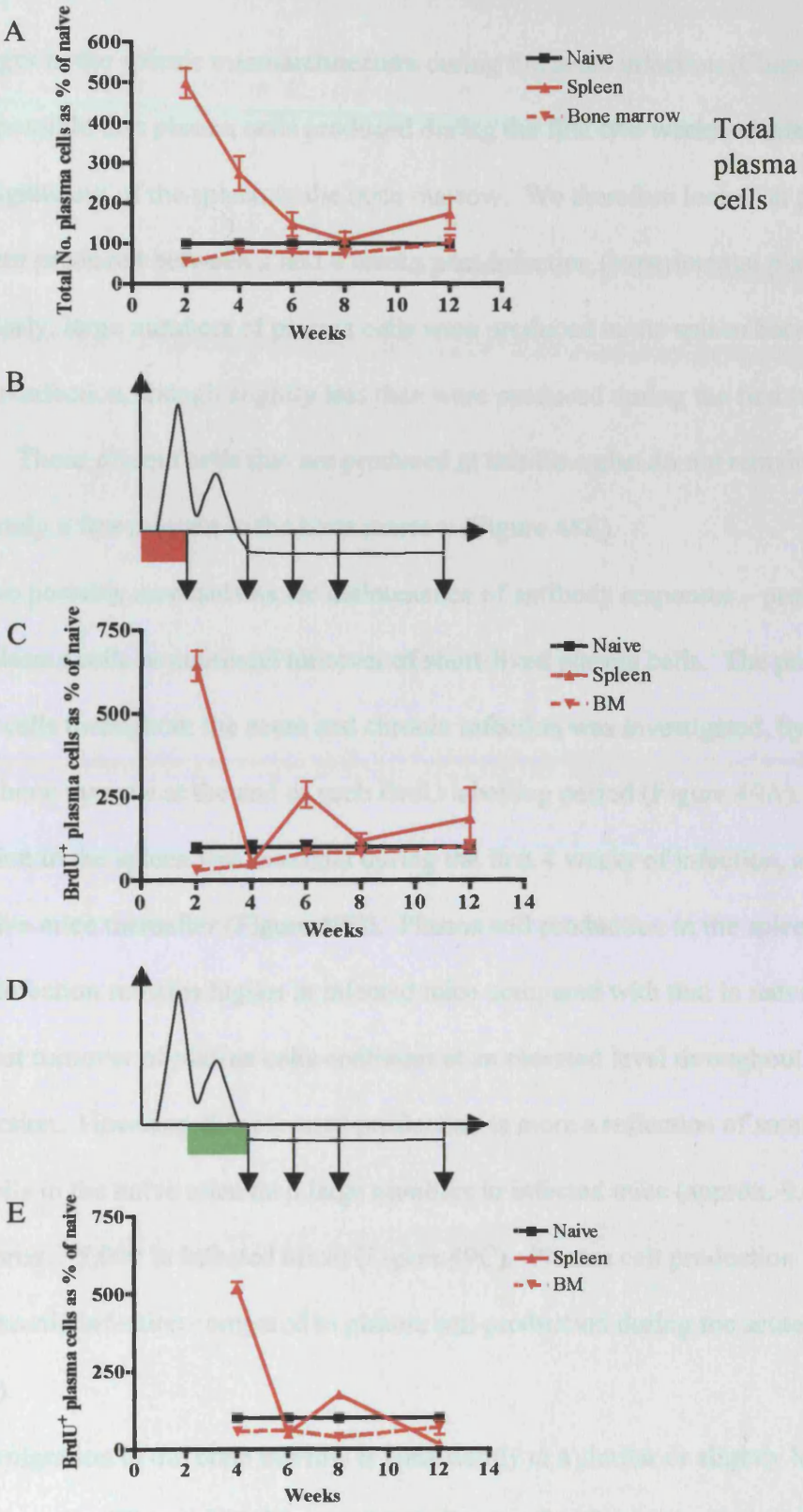
B) Experimental plan to show the period of BrdU labelling ( ) and sampling time points ( ) for spleen and bone marrow during infection

C) Total number of BrdU labelled plasma cells in spleen (–) and bone marrow (--) relative to naïve controls

D) Experimental plan to show the period of BrdU labelling ( ) and sampling time points ( ) for spleen and bone marrow during infection

E) Total number of BrdU labelled plasma cells in spleen (–) and bone marrow (--) relative to naïve controls

Points and bars represent the mean value +/- SEM of 3 age matched naïve controls and 4-5 infected mice per time point, and the horizontal black line indicates the normalised naïve controls (100%)


A
Total No. plasma cells as % of naive
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14
Weeks
Naive
Spleen
Bone marrow
Total plasma cells
B
C
BrdU+ plasma cells as % of naive
750
500
250
0
0 2 4 6 8 10 12 14
Weeks
Naive
Spleen
BM
D
E
BrdU+ plasma cells as % of naive
750
500
250
0
0 2 4 6 8 10 12 14
Weeks
Naive
Spleen
BM

Due to changes in the splenic microarchitecture during the acute infection (Chapter 4, [346]), it is possible that plasma cells produced during the first two weeks of infection are unable to migrate out of the spleen to the bone marrow. We therefore looked at plasma cells that were produced between 2 and 4 weeks post-infection (experimental plan Figure 48D). Similarly, large numbers of plasma cells were produced in the spleen between 2 and 4 weeks post-infection, though slightly less than were produced during the first two weeks of infection. Those plasma cells that are produced at this time also do not remain in the spleen, and only a few migrate to the bone marrow (Figure 48E).

There are two possible mechanisms for maintenance of antibody responses – production of long-lived plasma cells or continual turnover of short-lived plasma cells. The production of new plasma cells throughout the acute and chronic infection was investigated, by analysing spleens and bone marrow at the end of each BrdU labelling period (Figure 49A). Plasma cell production in the spleen was maximal during the first 4 weeks of infection, and similar to that in naïve mice thereafter (Figure 49B). Plasma cell production in the spleen at 12 weeks post-infection remains higher in infected mice compared with that in naïve mice, indicating that turnover of plasma cells continues at an elevated level throughout the chronic infection. However, this elevated production is more a reflection of small numbers of plasma cells in the naïve mice than large numbers in infected mice (approx. 9,000 in naïve cf. approx. 35,000 in infected mice) (Figure 49C). Plasma cell production is minimal during the chronic infection compared to plasma cell production during the acute infection (Figure 49B).

Plasma cell migration to the bone marrow is consistently at a similar or slightly lower level of that in naïve mice (Figure 49B, D), possibly indicating that fewer plasma cells

Figure 49. Turnover of plasma cells during *P.chabaudi* infection and contribution of plasma cells formed throughout infection to plasma cell pool at 12 weeks post-infection

A) Experimental plan to show the period of BrdU labelling; 0-2 weeks ( ), 2-4 weeks ( ), 4-6 weeks ( ), 6-8 weeks ( ) & 8-12 weeks ( ) post-infection; and sampling time points ( ) for spleen and bone marrow during infection

B) Relative number of BrdU labelled plasma cells in spleen ( ) and bone marrow ( ) as a percentage of plasma cell numbers in naïve controls

C) Absolute number of BrdU labelled plasma cells in naïve ( ) and infected ( ) spleen

D) Absolute number of BrdU labelled plasma cells in naïve ( ) and infected ( ) bone marrow (2 femurs)

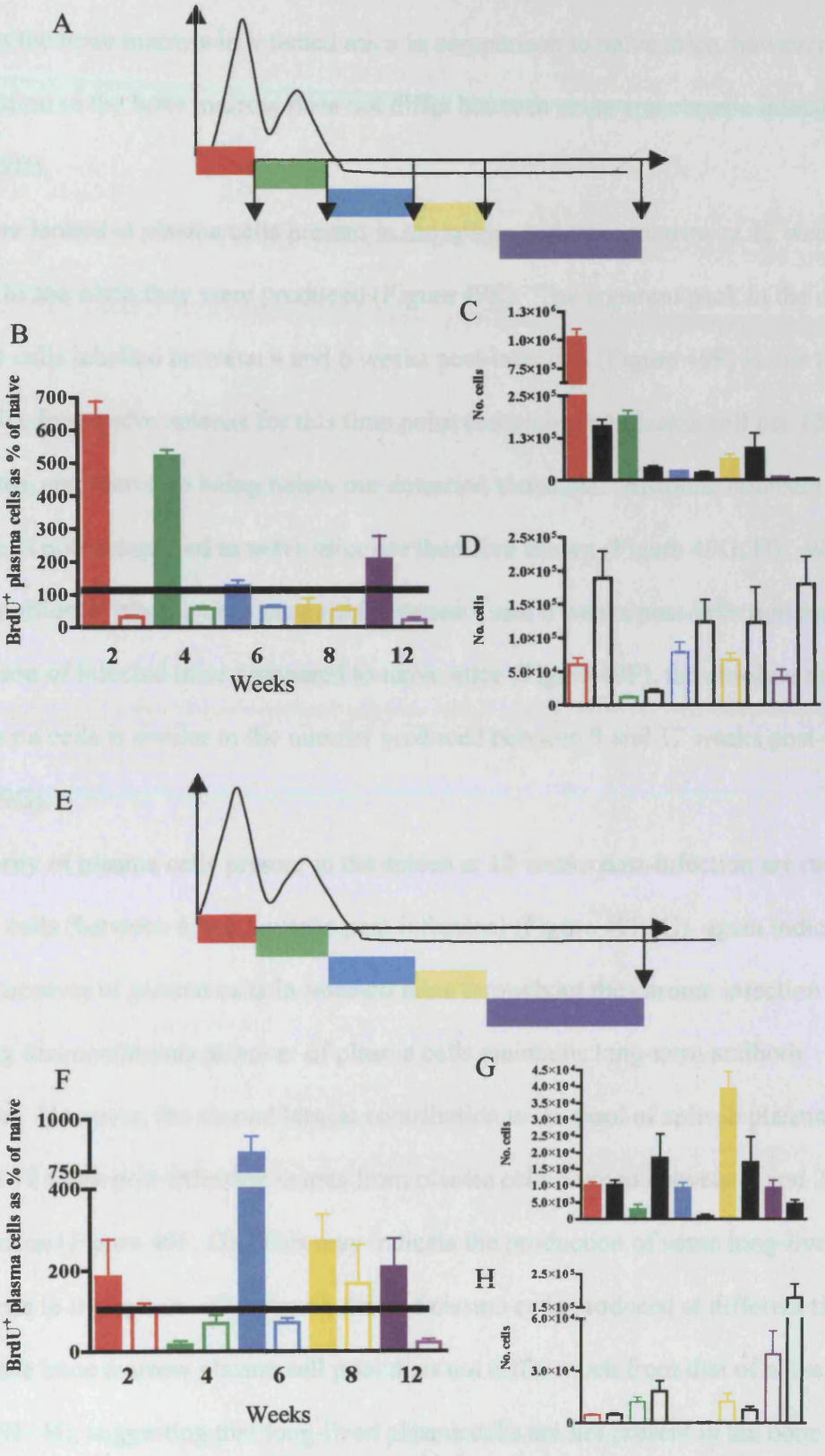
E) Experimental plan to show the period of BrdU labelling; 0-2 weeks ( ), 2-4 weeks ( ), 4-6 weeks ( ), 6-8 weeks ( ) & 8-12 weeks ( ) post-infection; and sampling time point ( ) for spleen and bone marrow at 12 weeks post-infection

F) Relative number of BrdU labelled plasma cells in spleen ( ) and bone marrow ( ) as a percentage of plasma cell numbers in naïve controls

G) Absolute number of BrdU labelled plasma cells in naïve ( ) and infected ( ) spleen

H) Absolute number of BrdU labelled plasma cells in naive ( ) and infected ( ) bone marrow (2 femurs)

Bars represent the mean value +/- SEM of 3 age matched naïve controls and 4-5 infected mice per time point, the horizontal black line indicates normalised naïve controls (100%)


A
B
BrdU+ plasma cells as % of naive
700
600
500
400
300
200
100
0
2
4
6
8
12
Weeks
C
No. cells
1.3×10⁶
1.0×10⁶
7.5×10⁵
2.5×10⁵
1.3×10⁵
0
D
No. cells
2.5×10⁵
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
E
F
BrdU+ plasma cells as % of naive
1000
750
400
200
0
2
4
6
8
12
Weeks
G
No. cells
4.5×10⁴
4.0×10⁴
3.5×10⁴
3.0×10⁴
2.5×10⁴
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
H
No. cells
2.5×10⁵
1.5×10⁵
6.0×10⁴
3.0×10⁴
0

migrate to the bone marrow in infected mice in comparison to naïve mice, however plasma cell migration to the bone marrow does not differ between acute and chronic infection (Figure 49B).

Finally, we looked at plasma cells present in the spleen and bone marrow at 12 weeks post-infection to see when they were produced (Figure 49E). The apparent peak in the number of plasma cells labelled between 4 and 6 weeks post-infection (Figure 49F) is due to half the samples from naïve spleens for this time point containing <1 plasma cell per 10^6 splenocytes, and therefore being below our detection threshold. Absolute numbers of plasma cells not normalised to naïve mice are therefore shown (Figure 49G, H). Whilst a large proportion of plasma cells produced between 4 and 6 weeks post-infection are present in the spleen of infected mice compared to naïve mice (Figure 49F), the absolute number of these plasma cells is similar to the number produced between 8 and 12 weeks post-infection (Figure 49G).

The majority of plasma cells present in the spleen at 12 weeks post-infection are recently produced cells (between 6 and 8 weeks post-infection) (Figure 49F, G), again indicating elevated turnover of plasma cells in infected mice throughout the chronic infection and suggesting that continuous turnover of plasma cells maintains long-term antibody production. However, the second largest contribution to the pool of splenic plasma cells present at 12 week post-infection comes from plasma cells formed between 0 and 2 weeks post-infection (Figure 49F, G). This may indicate the production of some long-lived plasma cells in the spleen. The contribution of plasma cells produced at different time points to the bone marrow plasma cell pool does not differ much from that of naïve mice (Figure 49F, H), suggesting that long-lived plasma cells are not present in the bone marrow

at 12 weeks post-infection, although we cannot exclude the possibility that the small number of plasma cells that do persist in the bone marrow are responsible, and sufficient, for long-term antibody production or that the anti-malaria serum antibody titre is not reflected by the number of long-lived plasma cells in the bone marrow.

Fluorescent labelling of recombinant malaria proteins

In order to compare the kinetics and longevity of malaria-specific cells to total B cells and plasma cells, attempts were made to detect malaria antigen-specific B cells and plasma cells by flow cytometry. To visualise malaria-specific cells, recombinant malaria proteins were labelled with fluorescent moieities. The recombinant MSP-1 proteins and their location in the intact MSP-1 molecule are shown in Figure 50.

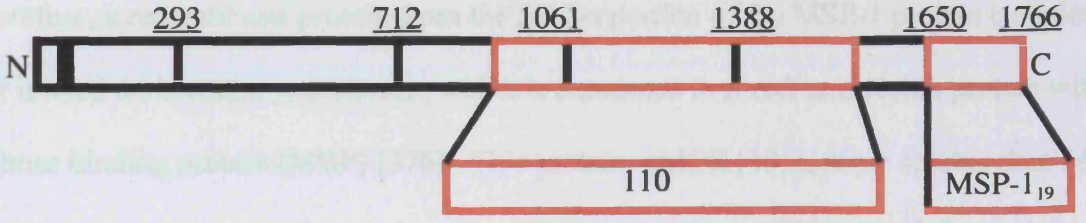


Figure 50. Full length *P.chabaudi* MSP-1 molecule and recombinant proteins. Full length protein (■), and two recombinant proteins [375, 376], that were labelled with fluorescent probes for detection of antigen-specific B cells and plasma cells (□). Numbers underlined indicate location of natural proteolytic cleavage sites. Figure adapted from [377]

Firstly, recombinant *P.chabaudi* MSP-1_{19} was expressed in *Pichia pastoris* with a histidine tag for purification [375], and was labelled with Alexa Fluor 647. The ideal fluorescent

dye to protein (F:P) ratio for flow cytometry analysis is thought to be approximately 5-6:1 [494], in order to obtain a good signal above background. This is especially important when dealing with low numbers of cells, such as expected for antigen-specific cells [495]. Alexa Fluor 647 labelling of MSP-1_{19} gave an F:P ratio of no greater than 1:1, and we were unable to detect MSP-1_{19} specific cells in flow cytometry analysis of MSP-1_{19} immunised mice. The low labelling efficiency may be due to the structure of the C-terminus of MSP-1. Structural analysis has shown that MSP-1_{19} is made of up two epidermal growth factor (EGF)-like domains with a C-terminal glycosyl phosphatidyl inositol (GPI) membrane anchor [358]. Unfortunately this means that there is a scarcity of lysine residues available on the surface, which provide the amine group that is essential for succinimidyl ester linkage of fluorescent moieties [494].

Therefore, a recombinant protein from the 38kDa portion of the MSP-1 protein complex that is shed on invasion was chosen, which is expressed in *E.coli* as a fusion protein with maltose binding protein (MBP) [376]. This protein, pMCK110, is more soluble than MSP-1_{19}, has a more open structure and is also more immunogenic [377]. Crucially, maltose binding protein provides more available lysine residues on the surface, which should provide a vehicle for better fluorescent labelling.

The F:P ratio obtained for Alexa Fluor 647 labelling of pMCK110 was approximately twice that obtained with MSP-1_{19}, at 1.8:1, which, although not ideal, is the highest degree of labelling that could be obtained with the recombinant MSP-1 proteins available. Chicken gammaglobulin (CGG) was used as a control protein, and labelled with an F:P ratio of 3:1.

Detection of antigen-specific cells

In order to test the feasibility of detecting antigen-specific cells, splenic B cells and plasma cells were analysed by flow cytometry 10-14 days after primary (CGG) or tertiary (pMCK110) immunisation. A low frequency of antigen-specific cells could be detected (Figure 51), however this was barely above that in naïve mice, and antigen-specific staining was not always greater than staining with the irrelevant control antigen. Double staining of antigen-specific cells with the same protein labelled with two different fluorophores, known as single epitope multi-staining (SEMS) [495], and pre-incubation of biotinylated protein with streptavidin to form biotin-streptavidin tetramers [268], have been reported to reduce background staining and allow clearer visualisation of antigen-specific B cells. Neither of these methods, however, were successful in allowing clear and reproducible identification of antigen-specific B cells and plasma cells in our hands.

Figure 51. Feasibility of detecting antigen-specific cells by flow cytometry

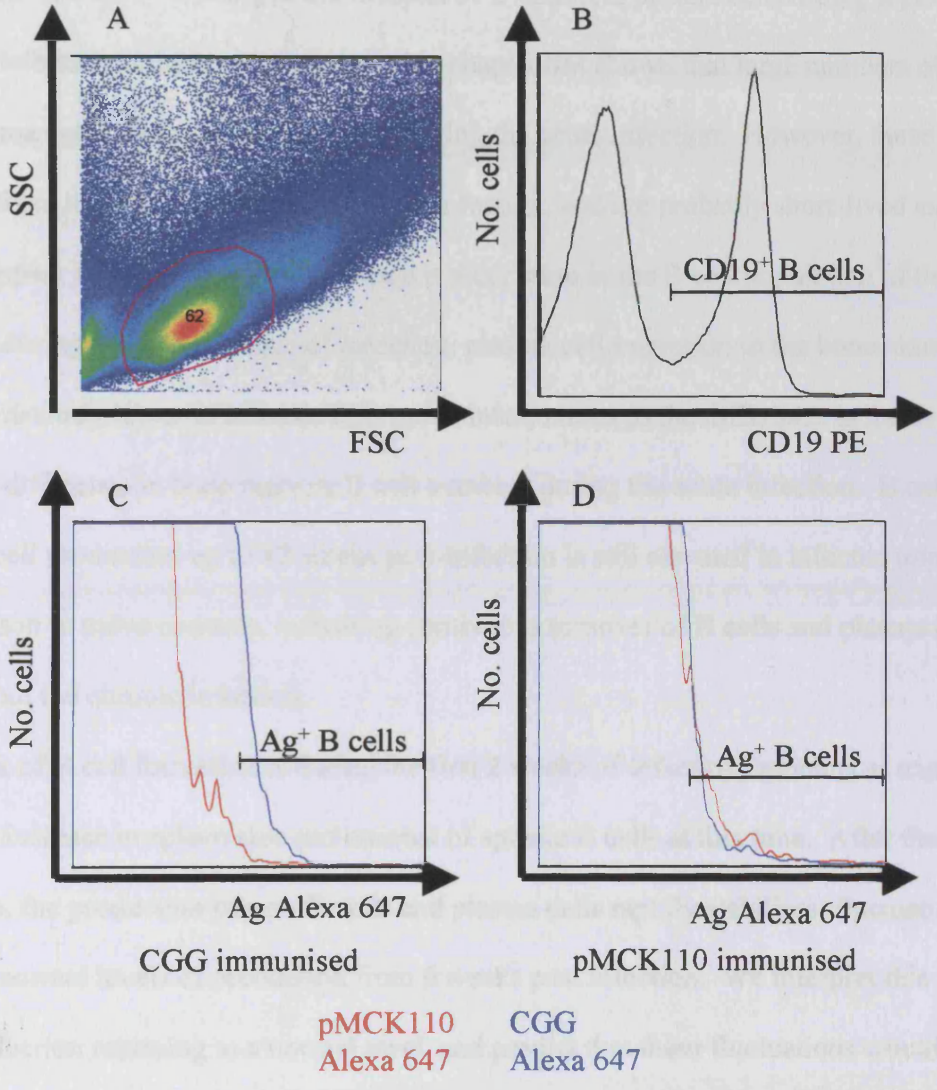
Spleens were taken from mice 10 days after primary immunisation with CGG or 14 days after tertiary immunisation with pMCK110. Approximately 5 x 10^5 cells were collected per sample.

A) FSC SSC profile showing live cell gate (–)

B) Single colour histogram showing $CD19^+$ B cells

C) Single colour histogram of viable $CD19^+$ B cells from CGG immunised mice stained with CGG-Alexa 647 (–) or with irrelevant Alexa 647 labelled protein (pMCK110) (–)

D) Single colour histogram of viable $CD19^+$ B cells from pMCK110 immunised mice stained with pMCK110-Alexa 647 (–) or with irrelevant Alexa 647 labelled protein (CGG) (–)


A
SSC
FSC
62
B
No. cells
CD19+ B cells
CD19 PE
C
No. cells
Ag+ B cells
Ag Alexa 647
CGG immunised
D
No. cells
Ag+ B cells
Ag Alexa 647
pMCK110 immunised
pMCK110
Alexa 647
CGG
Alexa 647

Discussion

This is the first study looking at the lifespan of B cells and plasma cells during a primary malaria infection in mice. The work in this chapter has shown that large numbers of B cells and plasma cells are found in the spleen during the acute infection. However, these cells are lost from the spleen during the chronic infection, and are probably short-lived as they do not appear in the bone marrow. There is a decrease in the B cell population of the bone marrow during the first 4 weeks of infection; plasma cell migration to the bone marrow is also consistently lower in infected than naïve mice, although the difference is much smaller than the difference in bone marrow B cell numbers during the acute infection. B cell and plasma cell production up to 12 weeks post-infection is still elevated in infected mice in comparison to naïve controls, indicating continuous turnover of B cells and plasma cells throughout the chronic infection.

The peak of B cell formation is during the first 2 weeks of infection, which is as expected with the increase in spleen size and number of splenic B cells at this time. After the peak of infection, the production of new B cells and plasma cells rapidly stabilises, fluctuating close to normal levels of production from 6 weeks post infection. We interpret this as B cell production returning to a normal level, and predict that these fluctuations would diminish after the clearance of chronic infection, until there is no difference between naïve and infected mice. It is interesting to note, however, that the alterations in B cell formation occur mostly during the acute infection, and that the homeostatic situation is mostly regained before residual parasitaemia is completely cleared.

The majority of B cells that undergo cell division during the first 2 weeks of infection do not persist long as $CD19^+$ B cells. Large numbers of these cells have been shown to undergo apoptosis [392], however others may have undergone further cell division, forming germinal centres and more plasma cells. We have shown that the loss of those B cells that divided during the first two weeks of infection from the spleen cannot be accounted for by migration of B cells to the bone marrow, by further cell division or by differentiation into plasma cells, indicating that the majority of these cells are indeed lost. Staining with an early apoptotic marker would confirm whether a large fraction of these B cells have died. For the majority of the period of observation, B cell production in the spleen remained elevated above the level of production in naïve mice. It is also clear that the majority of splenic B cells present at 12 weeks post-infection were recently produced cells, that divided between 6 and 12 weeks post-infection. This suggests that B cell turnover may be independent of the presence of live parasites, which are eliminated between 8 and 12 weeks post-infection, although a stimulatory effect of residual parasite antigen cannot be excluded. It will be necessary to look beyond 12 weeks post-infection to determine whether B cell production continues at this elevated level for long after the clearance of live parasites.

If B cells are continually turning over for the entire 12 weeks of the infection, it raises the question of whether long-lived memory B cells are produced, when they may be produced, and how they are maintained. The mechanism by which antigen-experienced B cells are maintained has been controversial for some time (review [496]). Gray & Skarvall [399] showed that after adoptive transfer of memory B cells into an irradiated antigen-inexperienced host, no memory response could be elicited after 12 weeks. They concluded

that the persistence of antigen was essential for the maintenance of memory B cells, and that in the absence of antigen, memory B cells have a half-life of 2-3 weeks. Memory cells that maintain themselves by continuous turnover would be more likely to require antigen-persistence than long-lived, non-cycling, memory B cells, and it is thought that persisting antigen would be maintained on follicular dendritic cells, although it is not know long antigen can be maintained on FDCs for. Antigen binding memory B cells, however, be seen in the marginal zone for up to a year after immunisation [207]. More recent work has argued that memory B cells can persist in the absence of antigen [248], and that survival of memory B cells does not require the persistence of antigen on FDCs [201].

In *P.falciparum* exposed humans with antibody responses to specific malarial antigens, memory B cells to those malarial antigens are not necessarily detectable in peripheral blood [400], suggesting that not all malaria infections resulted in the production of long-lived memory B cells. Similar observations have been made in some individuals after immunisation with tetanus toxoid [386]. These findings are in line with our experimental data on the total B cell pool, however it would be important to extend our work to the analysis of malaria-specific B cells.

A study of malaria-specific memory B cell responses [367] induced by vaccination of mice concluded that subsequent infection with *P.yoelii* induced deletion of malaria-specific memory B cells and long-lived plasma cells. However, the cell surface molecule, CD27, was used to identify memory B cells in this study. Whilst this has been shown to be a memory B cells marker in humans [237, 485] it is not clear whether it similarly detects memory B cells in mice [240]. Further functional studies would be needed to verify these findings.

The study of memory B cell production is hampered by the lack of a definitive cell surface molecule specific for memory B cells. We had hoped to bypass this by using fluorescently labelled antigen to identify malaria-specific B cells. Two major problems were encountered with this method. Firstly, the recombinant protein originally used, the C-terminal fragment of MSP-1 (MSP-1_{19}), was difficult to label with any fluorescent probe. This was probably due to its highly disulphide bonded globular structure [358], with few lysine residues available for succinimidyl ester linkage to the fluorescent probe. The second recombinant protein, from another part of MSP-1 (MSP-1_{38}, encoded by the plasmid pMCK110), was a fusion protein with maltose binding protein that had a more open structure with more lysine residues available. This protein was more readily labelled, however we were unable to obtain reliable, repeatable staining of plasma cells or memory B cells. The reason for the lack of visualisation of MSP-1 specific cells is likely to be the relatively low frequency of cells specific for any surface antigen of *P.chabaudi.* Previous studies from this lab have shown by limiting dilution assays that B cells specific for a crude mixture of malaria antigens was approximately 1 in 7,000 in the spleen [497], and ELISPOT analysis indicates that the frequency of MSP-1_{19}-specific memory B cells is approximately 1 in 80,000 during a primary infection (F. Ndungu, personal communication). At such a low frequency it would be very difficult to detect these cells by flow cytometry. The majority of reports describing flow cytometry analysis of antigen-specific B cells are from BCR transgenic mice which have a higher frequency of antigen-specific B cells [495], or in the response to NP ((4-hydroxy-5-nitrophenyl) acetyl) [498-500] where the frequency of NP-specific B cells is approximately 1 in 15-20,000 [501], compared to 1 in 80,000 for MSP-1_{19}, rendering FACS analysis of these cells more

feasible. A malaria-specific BCR transgenic mouse would be an ideal tool for the measurement of antigen-specific B cell responses during *P.chabaudi* infection.

Given the low number of malaria-specific memory B cells present in the spleen, and the improbability of detecting these by flow cytometry, we can say little about the production and maintenance of malaria-specific B cells. Although we have not been able to definitively identify malaria-specific memory B cells, our work on total B cell production and turnover suggests that continual turnover of B cells is a more likely mechanism for maintenance of memory B cells than the production of long-lived memory B cells during the 12 weeks of primary *P.chabaudi* infection. Further work, however, would be required to give a more definitive answer to this question.

The low level of B cell production in the bone marrow of infected mice compared with naïve mice during the first 2 weeks of infection was unexpected. B cell production in the bone marrow recovers quickly, however, and has returned to normal levels by 6 weeks post-infection. Previous studies have documented a decrease in cellularity in the bone marrow during acute malaria infection [342-344], although most studies on the composition of the bone marrow during malaria infection have focused on erythropoietic cells rather than on lymphoid cells.

There are two possible explanations for the lower numbers of B cells in the bone marrow during acute infection. The first is that all newly produced B cells may be quickly exported from the bone marrow to the spleen in response to the infection. This hypothesis assumes that export of B cells from the bone marrow can be increased, which is supported by the increased number of B cells observed in the spleen at this time. Regulation of B cell production in the bone marrow appears to be independent of feedback from the mature B

cell pool – i.e. proliferation of B cell progenitors is not affected by the number of mature B cells [502], therefore B cell production in the bone marrow should not be reduced as a consequence of large numbers of B cells in the spleen. B cell proliferation can, however, be prevented by elimination of macrophages by silica injection and by splenectomy [502], therefore the loss of macrophages from the marginal zone (Chapter 4) may be directly related to the reduction in the number of bone marrow B cells. It would be interesting to discover whether the presence of macrophages in the marginal zone and the level of B cell production in the bone marrow are causally related, or whether they are both symptoms of an unknown underlying cause.

The second possibility is that the high levels of pro-inflammatory cytokines in the blood during the initial stages of malaria infection suppress B cell production in the bone marrow. Levels of IL-1 in particular have been shown to suppress B cell production in the bone marrow [503], which may be worth investigating further. In agreement with this, bone marrow cellularity has been shown to decrease by up to 80% during acute malaria infection (D. Brown *et al*, in preparation) [342-344]. These two possibilities are not mutually exclusive, and the decline in the number of B cells in the bone marrow at 2 weeks post-infection could be due to a combination of increased export and decreased production of B cells.

Suppression of B cell production in the bone marrow has been demonstrated in several bacterial, viral and parasitic infection [504-506], and in influenza infection was shown to be due to apoptosis of early B cells mediated by TNFα/LTα [507]. This has been hypothesised to be responsible for the poor development of immunity to subsequent heterologous infections (e.g. influenza) [504]. Large amounts of TNFα/LTα are produced

during acute malaria infection [151, 172, 259], therefore suppression of B cell production by TNFα/LTα may be an important mechanism in malaria-induced immunosuppression during acute infection. This would, however, probably affect only immune responses to antigens encountered during the acute infection, and should not affect immune responses to antigens encountered at other times (e.g. to vaccination). TNFα/LTα is, however, important for memory responses, as mice lacking the TNFRI undergo a similar course of infection during secondary malaria infection as in primary infection [147]. A balance is therefore necessary, between the levels of TNFα/LTα required for the production of memory responses, and over-production of TNFα/LTα that causes suppression of B cell production in the bone marrow. Interestingly, bone marrow B cell production is also suppressed during pregnancy [508], which may be one reason for the increased susceptibility of pregnant women to malaria infection [509].

In addition to short-lived B cells in *P.chabaudi* infection, the majority of plasma cells produced during the first 4 weeks of infection are also short-lived. This result was expected, as it is highly unlikely that either the spleen or the bone marrow would have the capacity to support such a large number of plasma cells for long. It is not known, however, how many plasma cells the bone marrow is capable of supporting. Despite the increase in plasma cell numbers, there is little change in migration of plasma cells to the bone marrow at any of the time points investigated, suggesting that plasma cell migration to the bone marrow may be inhibited during malaria infection. Expansion of plasma cells in the *P.chabaudi* infection co-incides with a large inflammatory response in the spleen [151, 259], which may help to retain plasma cells in the spleen, as it has been shown that plasma cells produced in an inflammation-associated immune response remain at the site of

inflammation rather than migrating to the bone marrow [510]. Alternatively, the extent of changes observed in the splenic microarchitecture during the acute malaria infection (Chapter 4) may prevent plasma cells from migrating to the bone marrow. Although the changes in the splenic microarchitecture are reversed by 8 weeks post-infection [346], plasma cells are only responsive to migratory chemokines for 2 weeks after formation [488].

Previous work in our laboratory has shown that the total number of plasma cells resident in the spleen during acute *P.chabaudi* infection is much greater than that produced after immunisation with CGG [346]. Here we show that plasma cell numbers in the bone marrow are somewhat lower during acute malaria infection than in naïve mice. There is very little information available on total plasma cell numbers in other infections or immunisations, however there are reports on antigen-specific plasma cell responses in the spleen and bone marrow.

Total numbers of plasma cells per two femurs range from 2-4 x 10^5 cells in naïve mice, consistently slightly lower in malaria infected mice. In a study of virus-specific plasma cells, [231] showed that approximately 2,500 virus-specific plasma cells per two femurs could be seen in total bone marrow by day 15 post-infection, and that the number of virus-specific plasma cells in the bone marrow remained between 2,500-3,800 per two femurs up to day 103. This indicates that approximately 1% of bone marrow plasma cells are specific for one particular antigen. If we therefore assume that antigen-specific plasma cells constitute 1% of the total number of plasma cells in infection or immunisation, it is possible to compare our data with other studies (Table 8).

Table 8. Comparison of estimated number of total plasma cells produced in response to infection or immunisation

Infection/ Immunisation	Total number of plasma cells	
	Spleen	Bone marrow (2 femurs)
Naïve mice	1×10^6	$2\text{-}4 \times 10^5$
P.chabaudi day 14	5×10^6	2×10^5
CGG day 10 [a)]	3.75×10^5	ND
LCMV day 8 [b)]	1×10^6	$4\text{-}5 \times 10^4$
Tetanus toxoid (2° imm. day 10) [c)]	$3\text{-}4 \times 10^6$	$1.5\text{-}2 \times 10^4$
Ovalbumin ~ day 7 [d)]	1.5×10^7	7×10^6
NP ~ day 7 [e)]	5×10^6	2×10^5

[a)] [395], [b)] [250], [c)] [450], [d)] [511], [e)] [149].
ND = not done

With the exception of the response to OVA immunisation, the numbers of plasma cells detected during acute malaria infection are within the range described by others during infection and immunisation.

Migration of newly formed plasma cells to the bone marrow does not appear to alter throughout acute and chronic infection, and is consistently lower than that in naïve mice. This contrasts with observations of LCMV-specific plasma cells, where it was shown that 80% of LCMV-specific plasma cells were present in the bone marrow by day 30 post-infection, and the the number of antigen-specific plasma cells in the bone marrow remained

at a relatively high level for up to 103 days [231]. Therefore, it seems that the situation in *P.chabaudi* infection is different, and our observations indicate that plasma cell migration to the bone marrow is defective in the malaria infection. Inhibition of plasma cell migration to the bone marrow could explain the lack of long-lived antibody responses seen to many malaria antigens in humans [177, 311, 313, 314, 470-472], and the drop in antibody titre at 4 weeks in *P.chabaudi* infection [396] (Figure 49).

The decay in the number of plasma cells in the spleen slightly precedes the reduction of anti-malaria IgG antibody responses between 1 and 2 months post-infection. Short-lived plasma cells in extrafollicular antibody responses been shown to have a half-life of 3-5 days [148], therefore between 2 and 4 weeks post-infection (discounting production of new plasma cells), plasma cell numbers would be reduced to between 3% and 12.5% of those present at 2 weeks post-infection. The decay of serum antibody titre lags behind that of plasma cell numbers, as serum γ-globulin has a half life of approximately 4 days at normal concentration [473]. Catabolism of γ-globulin is increased at the peak of infection and returns to normal by day 12-18 post-infection [476], therefore the anti-malaria IgG titre should mirror the reduction in numbers of plasma cells with a delay of approximately 4 days. Splenic plasma cell numbers return to numbers similar to that of naïve mice between 4 and 6 weeks post-infection. Likewise, the anti-malaria serum antibody titre drops linearly from 4 weeks post-infection, and continues to drop as the number of plasma cells in the spleen stabilises. This indicates that there is a large short-lived plasma cell and antibody response.

During the chronic phase of infection (4-12 weeks), it appears that no long-lived plasma cells are generated, as most of the plasma cells present in both the spleen and bone marrow

at 12 weeks post-infection are those that were produced from cells dividing between 6 and 12 weeks post infection. Plasma cell production in the spleen is also elevated above that seen in naïve animals at 12 weeks post-infection, therefore the low level long-lived antibody response observed in *P.chabaudi* infection could be the product of short-lived plasma cells continously turning over throughout the chronic infection. Whether the antibody response subsequent to clearance of chronic infection at 8-12 weeks post-infection is due to these short-lived plasma cells, or to long-lived plasma cells generated after clearance of parasitaemia, remains to be determined. The threshold number of plasma cells required to maintain a protective serum antibody response is not known, however the number of plasma cells and the low level of long-lived malaria-specific antibody is sufficient to provide protection against challenge infection, at least up to 6.5 months after primary infection, as mice exhibit good immunity to secondary *P.chabaudi* infection [396, 512].

Maintenance of the long-lived antibody response by continuous production of plasma cells could be verified by treating mice with cyclophosphamide, an alkylating agent and potent suppressor of B cell activity and antibody production [513] during the chronic infection. This treatment would prevent production of new plasma cells, but would not eliminate long-lived plasma cells that had already been formed [514]. If anti-malaria serum antibody titres drop after this treatment, this would demonstrate a requirement for continued production of plasma cells in order to maintain anti-malaria antibody production.

Antibody responses to immunisation commonly last for many years. In humans, antibodies and memory B cells to vaccines composed of attenuated viruses such as smallpox have been detected up to 75 years after immunisation [249, 250]. Not all responses to

vaccination are this long-lived, however, and some decay to low or pre-immunisation levels within 4 years (review [262]). Antibody responses in mice can also persist for long periods of time (review [515]). This contrasts with antibody responses of humans to malaria antigens, where some responses decay within months of infection [177, 311, 313, 314, 470-472].

The maintenance of serum antibody levels has been hypothesised to be due to either continual production of short-lived plasma cells from antigen-experienced B cells, or production of long-lived plasma cells. One mechanism for maintaining low levels of serum antibodies for a lifetime has been suggested to be polyclonal activation of memory B cells to produce plasma cells [516]. Whether or not a low level of plasma cell turnover is required for the maintenance of antibody responses is still under debate [515]. Our data so far provide no evidence that long-lived B cells or plasma cells are generated the first 12 weeks of primary *P.chabaudi* infection, contrary to observations in other infections [231].

In summary, we have shown that large numbers of short-lived B cells and plasma cells are produced during acute malaria infection, that there may be some defect in plasma cell migration to the bone marrow, that kinetics of plasma cell formation agree with kinetics of anti-malaria serum antibody titres, and that the likely mechanism for maintenance of long-term anti-malaria serum antibody titres is continuous turnover of B cells and plasma cells.

Future directions for this work should include the development of a transgenic system to investigate antigen-specific B cells and plasma cells, the extension of these studies to later timepoints in the primary infection and to include an investigation of B cell and plasma cell responses in secondary infection. ELISPOTs are a more sensitive assay for detecting small numbers of memory B cells and plasma cells, both total numbers of antigen-producing cells

and numbers of antigen-specific cells. These also have the advantage that they require no genetic modification of mice. Whilst a transgenic mouse with malaria-specific B cells has the advantage of greater numbers of antigen-specific cells for flow cytometry analysis, it is nevertheless not a natural situation and therefore care must be taken when drawing conclusions from such work. A study of plasma cell migration would also be useful, to determine if migration to the bone marrow is inhibited during primary malaria infection, as well as further investigation of suppression of B cell production during acute infection. A comparison of both total and antigen-specific B cell and plasma cell responses in malaria infection to other infections and to immunisation would also be informative, as the migration of long-lived plasma cells to the bone marrow in response to infection has not been investigated. We need to understand why long-lived B cells and plasma cell do not appear to be generated in response to malaria infection if we hope to be able to induce long-lived responses to malaria by vaccination.

Conclusions

After decades of research into immune responses to malaria infection, we still do not know how immunity to malaria is formed, or why it takes so long to develop in humans. Changes in the splenic microarchitecture have been observed previously [345, 346, 517], however as the first step in relating these changes to the development of the immune response, the alterations in the splenic microarchitecture during the acute infection were documented in detail.

Changes in the microarchitecture of the spleen are extensive, yet the significance of them in terms of benefit or harm to the host, and whether they are part of a normal or dysregulated immune response, is still unclear. It is entirely possible that the merging of the T and B cells zones is a normal physiological response to infection, allowing the cognate interaction between T and B cells, that is required for T-dependent antibody responses, to occur. This would agree with the observations that germinal centres are formed in response to malaria infection, and a strong anti-malaria IgG antibody response is produced. Whilst germinal centers are formed, it is however possible that they may not have formed the division between dark and light zones, as this division is dependent on the appropriate production of chemokines that are also required for maintenance of the splenic microarchitecture [156]. The correct formation of germinal centres in acute malaria infection, and the interactions between T, B and dendritic cells, should be investigated to determine whether these alterations are part of a normal physiological response to infection, or part of an inappropriate, dysregulated response.

The spleen is the main secondary lymphoid organ involved in the immune response to malaria infection, as because of the large amount of blood flowing through the spleen, the cells of the spleen are in close contact with the parasite in the blood. However, lymph nodes are also secondary lymphoid organs, yet little work has been done on their role in immune responses to malaria infection. We do not know what effect the absence on lymph nodes in the presence on the spleen would have on susceptibility to infection, and we also do not know how, or if, the lymph node architecture is altered during malaria infection, or if this phenomenon is unique to the spleen. Whilst it would be technically difficult to perform the surgery necessary to remove all lymph nodes from a mouse, an investigation of lymph node microarchitecture during acute malaria infection should be carried out.

The cause of rearrangement of the splenic microarchitecture, whether or not it is caused by a parasite antigen and is related to the level of parasitaemia, and the signalling pathways involved have also not been determined. A single injection of the TLR ligand LPS causes some of the same alterations in architecture seen in malaria infection [349]. Repeated injections of LPS or other TLR ligands, to mimic the proliferation of a replicating antigen, may cause changes in the splenic microarchitecture that further resemble those seen in malaria infection. Alternatively, infection of mice lacking TLRs may fail to produce these alterations in the microarchitecture, and such a result would indicate a direct role for TLRs in the mechanism of these changes. A study of splenic microarchitecture in a secondary malaria infection, where the parasitaemia and pathology are reduced, might also indicate whether these changes are related to parasite numbers.

We have hypothesised that the alterations in splenic microarchitecture are caused by lack of signalling through the LTβ signalling pathway, as our observations agree with many

observations made of splenic microarchitecture in LTβ KO mice [170]. We therefore treated mice with a lymphotoxin-β receptor agonist antibody to try and prevent the alterations in splenic microarchitecture from occurring. Although this was unsuccessful, the problem may have been due to technical difficulties such as insufficient antibody dose, and this hypothesis would therefore benefit from further investigation. Reduction of LTβ signalling has also been associated with increased susceptibility to *L.donovani* and viral infection, secondary to alterations in the lymphoid microarchitecture [518].

One key observation was that large numbers of plasma cells were found in the spleen shortly after the peak of infection, which did not persist long-term [346]. We wanted to know whether these cells were short-lived, or whether they migrated to the bone marrow to become long-lived plasma cells. We hypothesised that the majority of these plasma cells would be short-lived, with a small number of long-lived plasma cells colonising the bone marrow.

Our results agreed with this hypothesis, with some plasma cells produced during the malaria infection migrating to the bone marrow, and a few becoming long-lived plasma cells. However, this number is less than the number of plasma cells establishing in naïve mice, and also less than the optimal number of plasma cells calculated to be required to maintain long-term protective antibody responses [519]. This indicates that there may be some defect in plasma cell migration to the bone marrow, either in trapping plasmablasts in the spleen during their migration window, in production of bone marrow homing chemokines, or in plasmablast responsiveness to chemokines. Of these three possibilities, the first is most likely, as reorganisation of the spleen has already been demonstrated, and may well make it difficult for plasma cells to migrate out. As blood flows through the bone

marrow, pRBC may also be found in the bone marrow, and may thereby interfere with the production of chemokines by bone marrow stromal cells. Alternatively, the unusual environment of the reorganised spleen may either interfere with the appropriate expression of receptors for bone marrow chemokines, or may favour the production of splenic, rather than migratory, plasma cells. Future work on this could include a study of the responsiveness of plasma cells to migratory chemokines, the expression of chemokines by bone marrow stromal cells and of chemokine receptors by plasma cells.

The increased turnover of B cells and plasma cells in chronically infected mice in comparison to naïve mice was unexpected. Whether this turnover is dependent on live parasites or on antigen retained on FDC is an important question that should be addressed. This could be done by extending these experiments to later timepoints post-infection, and by cyclophosphamide treatment of mice (to eliminate plasmablasts but not mature plasma cells) after clearance of chronic infection [513], thereby establishing whether long-term antibody titres are maintained by mature plasma cells, or by continual cycling of plasmablasts. If continual, antigen dependent, cycling of B cells and plasmablasts is necessary to maintain long-term antibody titres against malaria antigens, any vaccine would need to be able to provide a stable, long-term repository of antigen in order to maintain protective antibody titres.

In order to study the effects of chronic infection, and the presence or absence of chronic infection, on the development of the immune response, we first needed to establish how long the chronic infection persists for, and this was done successfully. As many infections in malaria endemic areas are cleared early by drug treatment, it would be useful to investigate the differences in the development of the immune response in hosts that

undergo chronic infection compared to those which are drug cured. This could have major significance for the management of malaria infection, if elimination of infection by drug treatment adversely affects the development of the immune response.

Summary

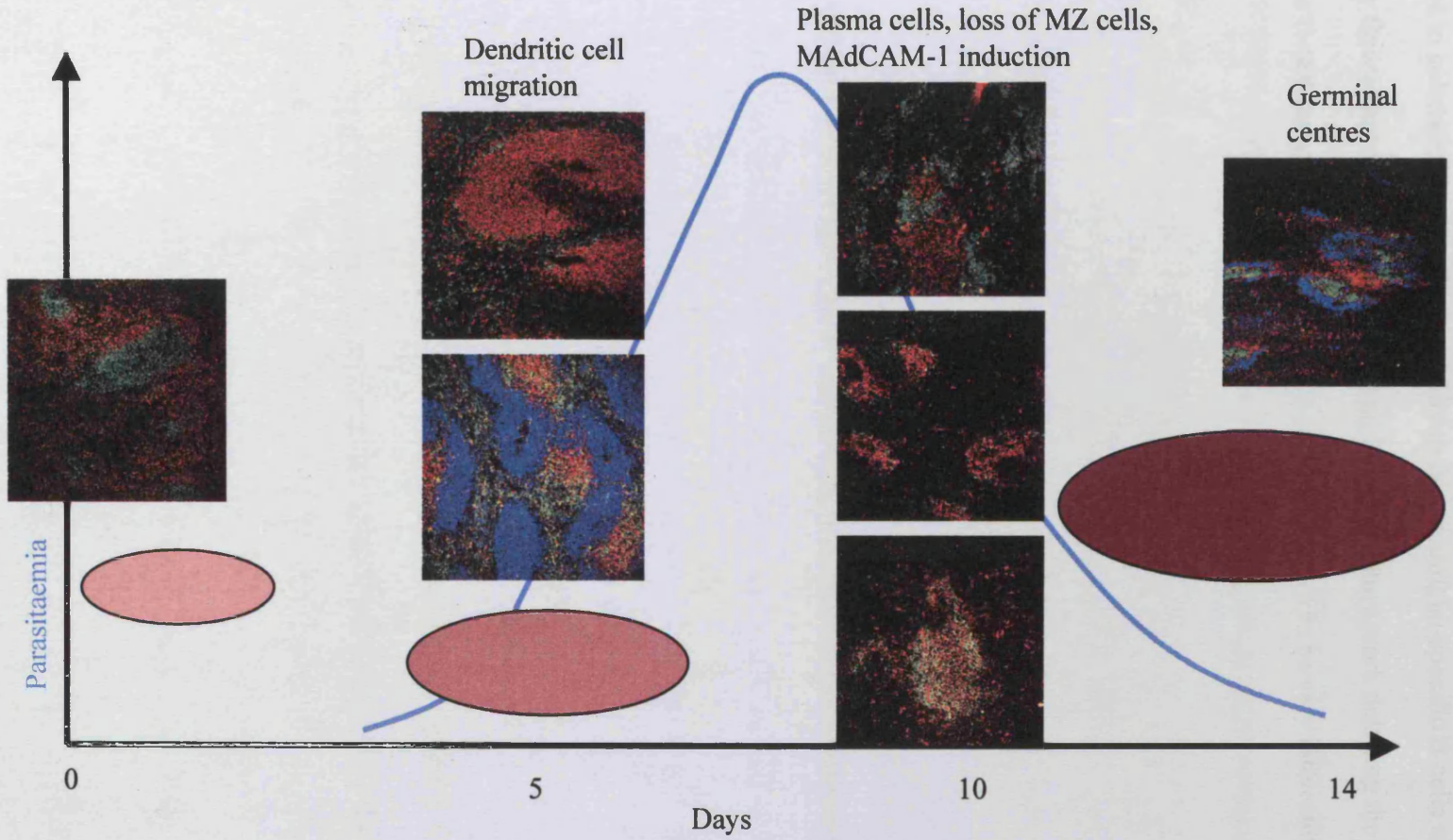
The extent of chronic *P.chabaudi* infection has now been established in two mouse strains. This is an important step to allow us to study the development of memory responses, the impact of chronic infection on the development of memory responses, the effect of chronic infection on immune responses to secondary malaria infection and the impact of chronic infection on immune responses to unrelated antigens. The difference in clearance of the chronic infection between the two mouse strains was unexpected, and raises interesting questions about how the speed of clearance of low-level infection is regulated, and about which response is better for the host. A comparison of the development of memory B cells, memory T cells and long-lived plasma cells between the two strains could be very informative.

The detailed analysis of the splenic microarchitecture allows us to pinpoint the order and timing of these changes. Whilst other investigators have previously described some of these alterations, we can now for the first time put them in sequence and closely follow their development. It is intriguing that whilst infected red blood cells can be seen in the spleen as little as 1 hour post-infection, it takes five days for the alterations in splenic microarchitecture to become visible. The first populations of cells to change their location are the dendritic cells, which is to be expected as these are the initiators of the adaptive immune response. This is swiftly followed by the beginning of the breakdown in separation between B and T cell zones, which is completed within the next 5 days. Marginal zone B cells and macrophages then migrate away from the marginal zone, as the expression of the adhesion molecule MAdCAM-1 expands to cover the white pulp area.

Finally, a large number of plasma cells can be seen in the spleen from day 8 post-infection (Figure 52). Despite all these alterations, there are some cell populations that are less affected. For example, red pulp macrophages do not alter their location, although they may increase in number.

The mechanism underlying these changes is an intriguing mystery. There is growing evidence that downregulation of chemokines is responsible for these changes [174, 179], however the initial trigger remains unknown. Such alterations to the splenic microarchitecture are not unique to malaria infection, and occur to a greater or lesser extent in other parasitic and viral infections [172, 174]. The lack of alterations during the first four days of infection, despite the presence of parasites in the spleen, indicates a threshold for the triggering of these changes, which may be temporarily self-perpetuating once they have begun. If parasite triggering of TLRs is responsible for initiating these changes, then it is logical that the number of parasites would need to increase before alterations were observed. A comparison of alterations in splenic microarchitecture between primary and secondary infection, or between low and normal inoculums of primary infection may shed some light on this.

It is not surprising that the majority of B cells and plasma cells produced during the acute infection are short-lived, however the reduction in B cell numbers in the bone marrow during acute infection, and the scarcity of long-lived plasma cells in the bone marrow was not predicted. Our results suggest that there may be suppression of B cell production in the bone marrow during acute infection, and that there may also be a defect in plasma cell migration to the bone marrow at this time. Our results also point towards the continuous turnover of B cells and formation of plasma cells throughout the chronic infection as a


Dendritic cell migration
Plasma cells, loss of MZ cells, MAdCAM-1 induction
Germinal centres
Parasitaemia
0
5
10
14
Days

mechanism for the maintenance of long-term antibody titres, although further work is required to substantiate this. The inability to detect antigen-specific B cells and plasma cells by flow cytometry was disappointing, however future work detailing the antigen-specific B cell response to malaria infection should prove to be very interesting.

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